NEGLECTED AND UNDER-RESEARCHED PARASITIC DISEASES OF VETERINARY AND ZOONOTIC INTEREST

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NEGLECTED AND UNDER-RESEARCHED PARASITIC DISEASES OF VETERINARY AND ZOONOTIC INTEREST

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EDITORIAL
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Editorial: Neglected and Under-Researched Parasitic Diseases of Veterinary and Zoonotic Interest

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Keywords: neglected parasitic disease, veterinary parasitic disease, zoonotic parasitic disease, parasites, zoonosis

Editorial on the Research Topic

Neglected and Under-Researched Parasitic Diseases of Veterinary and Zoonotic Interest

We are delighted to have contributed as Guest Editors to this special issue entitled: "Neglected and under-researched parasitic diseases of veterinary and zoonotic interest." This Special issue represents 17 manuscripts published from 94 authors (representing 17 countries) which, so far, encountered thousands views.

Neglected or poorly understood diseases can have a global impact as we have seen with COVID-19 reaching a global pandemic level very quickly. Interestingly, the parasitic diseases presented in this Special Issue are already having a human, animal, societal, and zootechnical impacts in their own rights but also sometimes due to the diseases that such parasites can carry, contributing to further morbidity and mortality.

In veterinary and human parasitology, many diseases have been excluded from international debate. Certain diseases are recognized as global emergencies, while others are considered niche or have dropped out of scientific interest. This imbalance may be due to two main reasons, which are independent from their health interest. First, the arbitration modalities of research funding and the evaluation of researchers (notably the mechanism of citation indices) in Western countries are such that they concentrate forces on a small number of parasites affecting the greatest number of people and tend to discourage studies on parasites that affect fewer people or people in countries located in other regions of the world, regardless of their health impact. Second, some parasites are elusive due to special lifestyles and thus largely unknown.

In this Special Issue you will learn about many neglected parasites (mites, protozoans, helminths) found in pets (cats and dogs), wildlife (chamoix), livestock animals (cattle, buffaloes, horses, poultry), and highlighting state-of-the-art techniques in potential drug targets or diagnostic markers. Some manuscripts are also highlighting the epidemiological aspects, and transmission risks for human populations as well.

This Research Topic discuss contributions on the parasitic diseases whose economic and health importance and spread is threatened due to the following reasons:

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- Underestimated due to their particular lifestyle that makes them elusive and difficult to study (e.g., poultry ectoparasites, including Dermanyssus gallinae and Ornithonyssus bursa and their zoonotic role);
- Erroneously considered eradicated (e.g., habronematidosis in equids);
- Lesser studied due to prevalence in low-income countries (e.g., Cryptosporidium spp., Sarcocystis in horses, Dracunculus in dogs);
- Often ignored (e.g., eimeriosis and neosporosis in ruminants, cestodosis in dogs) and in unexplored hosts (e.g., Eimeria in wild animals, Neospora and Toxoplasma in ducks);
- Rare and unrecognized (e.g., feline angiostrongylosis);
- Lack of/incorrect treatment (e.g., babesiosis in humans).

These neglected diseases should be taken into serious consideration. As we have seen with Covid-19, unknown, poorly studied or emerging diseases can have a global impact on humans, animals, and the environment and the neglected parasitic diseases presented in this Special Issue deserve further monitoring and research development.

We hope you will enjoy reading and reflecting on these important topics and consider challenging such knowledge gap while developing new integrated and multidisciplinary collaborations.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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A Novel Thioredoxin-Dependent Peroxiredoxin (TPx-Q) Plays an Important Role in Defense Against Oxidative Stress and Is a Possible Drug Target in *Babesia microti*

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Thioredoxin peroxidases (TPxs) are ubiquitous cysteine-based peroxidases that reduce peroxides as part of antioxidant defenses and redox signaling and are essential for *Babesia microti* protection against adverse environment agents like reactive oxygen species (ROS) and reactive nitrogen species (RNS). To better systematically understand TPxs, we identified a novel 2-Cys peroxiredoxin-Q (BmTPx-Q) of *B. microti*. The full-length BmTPx-Q gene is 653 bp that consists of an intact open reading frame of 594 bp that encodes a 197-amino acid protein. The predicted protein has a molecular weight of 22.3 kDa and an isoelectric point of 9.18. Moreover, BmTPx-Q showed low identity at the amino acid level to other peroxiredoxins (Prxs) among the currently known subfamilies. The recombinant BmTPx-Q protein (rBmTPx-Q) was expressed in *Escherichia coli* and purified with beads. The native protein BmTPx-Q was detected using mouse anti-BmTPx-Q polyclonal serum with western blotting and indirect immunofluorescence assay (IFA). In addition, enzyme activity was observed using nicotinamide adenine dinucleotide phosphate (NADPH) as substrate and triggered the NADPH-dependent reduction of the Trx/TrxR system. It was also discovered that BmTPx-Q mainly exists as a monomer whether under its native or functional states. In addition, when incubated with Chloroquine diphosphate salt for 24 h *in vitro*, the expression of BmTPx-Q showed a marked downward trend with the increase of drug concentration. These results suggest that *B. microti* uses BmTPx-Q to reduce and detoxify hydrogen peroxides to survive and proliferate inside the host. Furthermore, BmTPx-Q showed the lowest identity with host enzymes and could be a potential drug target for the development of novel strategies to control *B. microti* infection.

Keywords: *Babesia microti*, thioredoxin, peroxidase-Q, antioxidant activity, drug target

INTRODUCTION

Babesiosis is a serious disease caused by infection with protozoan parasite *Babesia microti* that is transmitted to humans via the bite of an infected tick or a contaminated blood transfusion. There have been many reports of cases from Europe and the USA in recent years (1–3). Babesiosis has a huge impact on elderly, splenectomized or immunocompromised patients, leading to anemia, fatigue, and fever hematuria (4). Although babesiosis can be controlled by treatment with antiparasitic drugs, many drugs have safety issues (5). Therefore, identification of new drug targets is needed to develop novel therapeutic strategies and overcome setbacks, such as drug resistance.

It is well-known that *Babesia* has a complex life cycle, including both arthropod vectors and mammalian hosts, and that it replicates in the host's red blood cells. Since it is surrounded by oxygen-rich environments, the parasite is likely to counteract the toxic effects of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that could induce oxidative DNA damage and lipid peroxidation (6, 7). The ROS and RNS are highly active compounds during normal cell metabolism. Therefore, to avoid the deleterious effects of ROS, various defense mechanisms have been adapted, such as non-enzymatic elements, which include glutathione (GSH) and vitamin C, and antioxidant enzymatic elements [i.e., catalase (CAT), superoxide dismutase (SOD), as well as peroxidase] (8). The parasite has developed a wide range of antioxidant systems, including peroxiredoxins (Prxs) (9), to keep their redox balance while living inside host erythrocytes (10, 11). Prxs have been a research topic of interest as a family of thiol-specific non-heme antioxidant peroxidases detoxifying hydrogen peroxide (H_2O_2), alkyl peroxides, and peroxynitrite (12). Moreover, Prxs are expressed at high levels in cells of almost all organisms, which protects cells against toxicity from ROS by reducing and detoxifying hydroperoxides, highlighting the importance of this protein family.

The Prx proteins have been divided into those that contain a single catalytic cysteine (Cys) residue and those that have an additional conserved residue (13). However, a new classification system has been suggested by Nelson and colleagues (14). Under this classification, Prxs are divided into six subfamilies based on the abundant sequence homology and structural similarity analyses, namely: Tpx, PrxQ-BCP, Prx1/AhpC, Prx5, Prx6, and AhpE (15–17). Alternatively, on the basis of the presence or relative locations of the resolving cysteine (Cr) residue, Prxs are classified into three types based on the distinct catalytic mechanisms: 1-Cys Prxs (Prx6 and AhpE), the typical 2-Cys Prx (Prx1/AhpC), and atypical 2-Cys (Tpx, TPx-Q, and Prx5) (18).

Prxs are collectively called thioredoxin peroxidases (TPxs) and constitute a large family of thiol-dependent peroxidases (18). Prxs are also potential drug targets. The studies demonstrated that these poorly cope with oxidative stress in Prx knockout strains of *Plasmodium falciparum* (19, 20). Recently, several TPxs of malaria parasites were identified, and the functional properties of the enzymes were considered key factors for the development of new drugs (21–24). Prx has also been reported to play key roles in innate immunity and inflammation (25) besides cellular redox signaling (26). Among these Prxs, the TPx-Q subfamily members have been proposed to be the most ancestral, most complex and the least systematically characterized (27). The TPx-Q proteins are thiol-based peroxidases, and are important for maintaining redox homeostasis in several organisms. TPx-Q is an atypical Prx that has already been identified in bacteria, parasites, and some lower eukaryotes but is not found in mammals (28–31).

TPx-Q, which is a cysteine-based peroxidase, has been detected in various protozoan parasites. TPx-Q-mediated resistance to major stresses largely relies on ROS degradation. TPx-Qs usually occurs at the monomeric state, with an intramolecular disulfide bond that are reduced by thioredoxins (Trx) (32). It has been shown that over-expression of TPx-Qs can cope with oxidative stresses in cyanobacteria. TPx-Q B from *Mycobacterium tuberculosis* is monomeric under reduced and oxidized states, and it is a thioredoxin-dependent and highly efficient fatty acid hydroperoxide reductase (33). Regardless of the mode of classification of these proteins, the catalytic mechanism of the enzyme remains central and relies on the redox-active cys, which is highly conserved in its amino acid sequence (34).

Although Prxs have been extensively studied, especially Tpx and Prx1 in *Plasmodium*, little is known about the Trx peroxidases-Q (TPx-Q) of *B. microti*. Sequencing of the full genome for *B. microti* has been completed (35), but the currently existing results are unilateral and unsystematic. In our previous study, we have done some basic research about the 2-Cys Trx peroxidases-1 and peroxidases-2 of *B. microti* (BmTPx-1 and BmTPx-2) (36, 37) and found evidence suggesting that the *B. microti* possesses at least two Prx subfamilies (Tpx and PrxQ). In this study, we identified and characterized a novel thioredoxin peroxidase (BmTPx-Q) from a strain of *B. microti*, analyzed the activity and assessed the BmTPx-Q expression after treatment with antiparasitic agents. All results suggest that *B. microti* can use BmTPx-Q to reduce and detoxify H_2O_2 for survival. Additionally, our new investigations on BmTPx-Q, a member of Prxs, provide new insights into the structure and function of Prx. We demonstrated that BmTPx-Q might act as an oxidative stress defensive molecule as well as drug target in *B. microti*.

MATERIALS AND METHODS

Parasite Culture *in vivo*

The ATCC® PRA-99TM strain of *B. microti* was obtained from the American Type Culture Collection (ATCC, USA) and maintained by serial passage in BALB/C mice (SLAC Laboratory Animal Co., Ltd., China) using a method described previously (36). The parasites were isolated until the erythrocyte infection rate reached 30%−40%, which was confirmed with Giemsa-stained thin-blood smears.

Parasite Culture and Treatment *in vitro*

For one independent experiment, *B. microti* was obtained from at least two mice; 3 days after infection (according to our protocol). The blood of the infected mice was carefully collected under aseptic conditions and added to bacteria-free anticoagulant. Then the blood was passed through a 27G needle three to five times. The blood cell debris was removed with a 5 μm syringe

filter, and most of the parasites were isolated. Subsequently, the parasites were centrifuged at 2,000 rpm for 5 min with a horizontal centrifuge. The supernatant was discarded and the pellets were re-suspended with sterile culture medium. The pellets used for incubation experiments were then washed three times with phosphate-buffered saline (PBS, pH 7.2) containing 50 μg/mL gentamycin sulfate (Sigma) under aseptic conditions. Healthy red blood cells were taken from 21-days-old normal mice and were distributed in a 12-well culture plate. Subsequently, each well was cultured in 5% CO_2 at 37°C in RPMI 1640 (Gibco, USA) supplemented with 1% penicillin/streptomycin (Gibco, USA) and 40% fetal bovine serum (Gibco, USA). The incubations were performed for 12 and 24 h at 37°C in a 5% CO_2 incubator.

Cloning and Sequence Analysis of *B. microti* TPx-Q (BmTPx-Q)

To find more novel genes from *B. microti*. Total RNA sequencing was performed to characterize all transcriptional activity. The brief method of preparing total RNA and cDNA for library construction and sequencing of the samples was described in our previous study (36). The full-length cDNA of the putative BmTPx-Q was generated by transcriptome analysis. The sequence was analyzed as described in our previous investigation (37). The Genetyx software (Software Development Co., Ltd., Tokyo, Japan) was used for the analysis of BmTPx-Q nucleotide and amino acid sequences. To further understand the relationships between BmTPx-Q and other TPx-Q genes, the inferred amino acid sequence of the BmTPx-Q was compared with other protein sequences (namely, the homologous TPx-Q proteins from different species) retrieved from the GenBank database using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/). The protein domain was identified using BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The signal peptide was predicted with the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/).

Expression and Purification of Recombinant BmTPx-Q (rBmTPx-Q)

The recombinant proteins (rBmTPx-Q) were produced as described previously (36). Briefly, the open reading frame (ORF) of BmTPx-Q was amplified by PCR with the following primer pair (forward) 5′-TT CAT ATG TTC AAA ATA CTG AAT TCA CGG-3′ and (reverse) 5′-TT CTCGAG CAG TTT ATC AAT AAA TTC-3′ (the underlined sequences contain the *Nde*I and *Xho*I restriction sites). The PCR product was inserted into the expression vector pET-30a (Novagen, USA). The recombinant plasmids harboring the BmTPx-Q (BmTPx-Q/pET-30a) coding sequence were transformed into *E. coli* (strain BL21). Induction of rBmTPx-Q Histidine-tag expression was performed using 1 mM isopropyl thio-b-D-galactoside (IPTG), followed by purification using Ni-NTA agarose beads (Merck-Millipore Corporation, USA). Purified rBmTPx-Q was evaluated a sample in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using reducing conditions and stained it with Coomassie Brilliant blue R-250. Protein concentration was determined with the BCA assay (Thermo Fisher Scientific, USA).

Antioxidant Activity Assay

To evaluate the antioxidant activity of rBmTPx-Q applying a mixed-function oxidation (MFO) assay (21, 29, 38). First, rBmTPx-Q (250, 500, and 1,000 μg/mL) was added to the reaction mixture, followed by incubation at 37°C for 1 h. Then, 500 ng of pBluescript SK(-) (Stratagene, USA) plasmid DNA was added to the reaction mixture, followed by incubation for an additional 2.5 h. Plasmid nicking was determined by using the MFO assay and analysis by 1% agarose gel electrophoresis, which was stained with DuRed dye (Fanbo Biochemicals Co. Ltd., China). Subsequently, to investigate whether rBmTPx-Q possesses peroxidase activity using a ferrithiocyanate system (39). The reaction mixtures (200 μL) containing rBmTPx-Q (1.5 μg protein) and 85 μL of buffer (0.5% glycerol/5 mM DTT/0.03 × PBS) was pre-incubated at room temperature (RT) for 2 min. H_2O_2 (1 mM) was used to start the reaction and was terminated with 40 μL of 26% trichloroacetic acid, which was added at 2-min intervals. The disappearance of H_2O_2 was monitored to assess rBmTPx-Q activity. In the reaction mixture, the remaining peroxide content was allowed to react with ∼40 μL of 10 mM $(NH_4)_2Fe(SO_4)_2$ and 20 μL of 2.5 M KSCN, which formed a ferric thiocyanate complex that was red in color. The color intensity was measured at a wavelength of 475 nm using a microplate reader (SpectraMax M5; Molecular Devices, USA). In the presence of Trx and Trx reductase (TrxR), oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) coupled with rBmTPx-Q to the reduction of H_2O_2 was examined using the method described by Kang et al. (40). NADPH oxidation was monitored in A_{340} in a 200-μL reaction mixture (0.14 μM TR, 6.4 μM Trx, 0.375 mM NADPH, 500 μg/mL rBmTPx-Q protein, 250 μM H_2O_2, 50 mM HEPES). The negative control (-)rBmTPx-Q indicates the absence of BmTPx-Q in reaction mixture.

Western Blotting for the Native BmTPx-Q Protein

To identify the native BmTPx-Q protein in the lysate of *B. microti*, *B. microti*-infected red blood cells (iRBCs) were prepared from Kunming mice at 4, 5, 6, 7, and 8 days post-infection, and non-infected erythrocytes were used as a negative control. First, the infected blood samples were centrifuged in low speed and hemolyzed by red cell lysis buffer (Tiangen Biotech, China) after discarding the supernatant. The soluble fractions (20 mg per lane) were separated on a 12% SDS-PAGE. The Western blot analysis was performed as described previously (36). Briefly, the proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Merck-Millipore Corporation, USA), and was blocked with 5% skim milk diluted in PBS/0.05% Tween (PBST) for 2 h at 37°C. Then, the membranes were incubated overnight at 4°C in mouse anti-rBmTPx-Q serum diluted to 1:200 in PBST. The blotted membranes were washed using PBST, followed by incubation in the presence of goat anti-mouse IgG antibody (horseradish peroxidase-conjugated; dilution, 1:2,000; Bethyl Laboratories, Inc., USA) for 1 h at 37°C. A washing step took place with PBST

before applying an enhanced DAB chromogenic substrate kit (Tiangen Biotech, China) to visualize the bands in accordance with the manufacturer's guidelines.

Expression Analysis of BmTPx-Q Post-infection

Assessment of BmTPx-Q expression post-infection was performed according to a method described elsewhere (41, 42). Briefly, we collected blood from mice infected with 1×10^8 iRBCs at 1–10 days post-injection, from which total RNAs were extracted using TRIzol. The cDNA was constructed by a PrimeScript RT reagent kit with gDNA eraser (TaKaRa, Japan) and used in quantitative real-time PCR (qRT-PCR) analysis. The qRT-PCR was performed with SYBR® Premix Ex Taq TM II (TaKaRa, Japan) and a StepOne Plus PCR system (Applied Biosystems). The primer pair of BmTPx-Q were BmTPxQ-qRT-F: ACAAGCACAATCTCCCATAC (forward) and BmTPxQ-qRT-R: TCTCCAGCACTAACTCCC (reverse). The 18s ribosomal RNA of *B. microti* (Bm18S) (GenBank: XM_021481625.1) was used as an internal control. The primer pair of Bm18S were Bm18S-qRT-F: GTTATAGTTTATTTGATGTTCGTTT (forward) and Bm18S-qRT-R: AAGCCATGCGATTCGCTAAT (reverse). The parasitemia was calculated at each day post-infection. Analysis was performed using the $2^{-\Delta\Delta ct}$ method, and experimental values were expressed as relative amounts (43).

Indirect Immunofluorescent Antibody Test

To determine the intracellular location of BmTPx-Q in *B. microti*, the test was performed as described previously (36). Briefly, thin smears of iRBCs were prepared and fixed in 50% acetone-50% methanol solution for 10 min at −30°C. Mouse antiserum against rBmTPx-Q was used as the primary antibody (dilution, 1:200), incubated at 37°C for 45 min. After it was washed with PBS, Alexa-Fluor® 488-conjugated goat anti-mouse IgG (Life Technologies Corporation, USA) was added as a secondary antibody (dilution, 1:2,000) and incubated at 37°C for 45 min. Then, the slides were washed using PBS and incubated with 0.5 μM 4′,6′-diamidino-2-phenylindole (Molecular Probes, Inc., USA) at RT for 20 min. The slides were later washed and mounted with Cytomation fluorescent mounting medium (Dako Corporation, USA) and visualized under a confocal laser-scanning microscope (Zeiss LSM 880, Germany).

Transcript Changes of BmTPx-Q After Treatment With Antiparasitic Agents

To assess the mRNA relative expression profile of BmTPx-Q after treatment with antiparasitic agents, a short-term culture system of *B. microti* iRBCs was established *in vitro*. This study was performed as described in previous research (41). Briefly, *B. microti*-infected mice blood (∼30% parasitemia) was collected and iRBCs were washed with PBS. A 12-well flat-bottom plate (Thermo, USA) was used for drug screening. The *B. microti* iRBCs ($\sim 2 \times 10^7$) were cultured in RPMI 1640 (Life Technologies, USA) supplemented with 25 mM HEPES (Life Technologies, USA) and 40% fetal bovine serum (Life Technologies, USA) at 37°C in a 5% CO_2 (44, 45). To evaluate the effect of drugs on BmTPx-Q gene expression, the iRBCs were grown *in vitro* for a short period before exposure to the three antiparasitic drugs (Quinine monohydrochloride dehydrate, Dihydroartemisinin, and Chloroquine diphosphate salt) (Sigma Aldrich, USA) was assessed. Quinine and Dihydroartemisinin were dissolved into dimethylsulfoxide (DMSO), while Chloroquine was dissolved in PBS. iRBCs were treated with various concentrations (20, 50, or 100 μM) of Quinine, Dihydroartemisinin, and Chloroquine at different timepoints (12, 24, and 36 h), meanwhile the controls were treated with DMSO or PBS. Relative BmTPx-Q transcript levels were assessed as previously described, by Reverse Transcription (RT)-PCR. Briefly, total RNA was isolated from infected RBCs using TRI solution (Life Technologies Corporation, USA), and RT-PCR was carried out applying 1 μg of total RNA and specific primers by a PrimeScript™ One-Step RT-PCR Kit (Takara, China). Conditions for the PCRs were as follows: 95°C for 30 s; 95°C for 5 s, 60°C for 35 s, for 40 cycles. The experiment was repeated in triplicate.

Statistical Analysis

A GraphPad PRISM 5 software (GraphPad Software Inc., CA, USA) was used for the data analysis. The mean ± standard deviation (SD) of each group was calculated. The differences between groups were assessed using two-tailed *t*-tests. $P < 0.05$ was considered significant and $P < 0.01$ was considered highly significant.

RESULTS

Identification and Characterization of the BmTPx-Q Gene

The identified full-length cDNA of BmTPx-Q has 653 bp in which includes a single ORF of 594 bp. Sequence analysis indicated that the ORF of BmTPx-Q gene encodes a protein of 197 amino acids with a theoretical molecular weight and isoelectric point of 22.3 kDa and 9.18, respectively. The amino acid sequence was deduced from the cDNA sequence of BmTPx-Q (**Figure 1A**). SignalP server indicated that BmTPx-Q has a signal peptide. The BmTPx-Q protein sequence was 98% identical to that of *B. microti* Prx Q (RI strain, XP_012647890.1), showing 35% sequence similarity with Prx Q of *Babesia bigemina* (XP_012767998.1), 35% with Prx Q of *Blastomyces dermatitidis* (EEQ83458.1), 39% with Prx Q of *Babesia* sp. *Xinjiang* (XP_028870106.1), and 43% with Ahp/TSA family-related protein, putative of *Theileria annulata* (XP_954500.1) (**Figure 1B**). The sequence alignment showed that the conserved peroxidatic cysteine (Cp) of BmTPx-Q is located at position 95 in a PxxxTxxC-motif, and an additional conserved cysteine at position 100 in BmTPx-Q (**Figure 1B**). The BmTPx-Q sequence contains a Thioredoxin-dependent hydroperoxide peroxidase activity of bacterioferritin comigratory protein (PRX-BCP) domain, the PRX-BCP is a new member of the thiol-specific antioxidant protein (TSA)/Alkyl hydroperoxide peroxidase C (AhpC) family (**Figure 1C**). The BmTPx-Q belonging to the Thioredoxin-like superfamily, it also contains a catalytic triad on

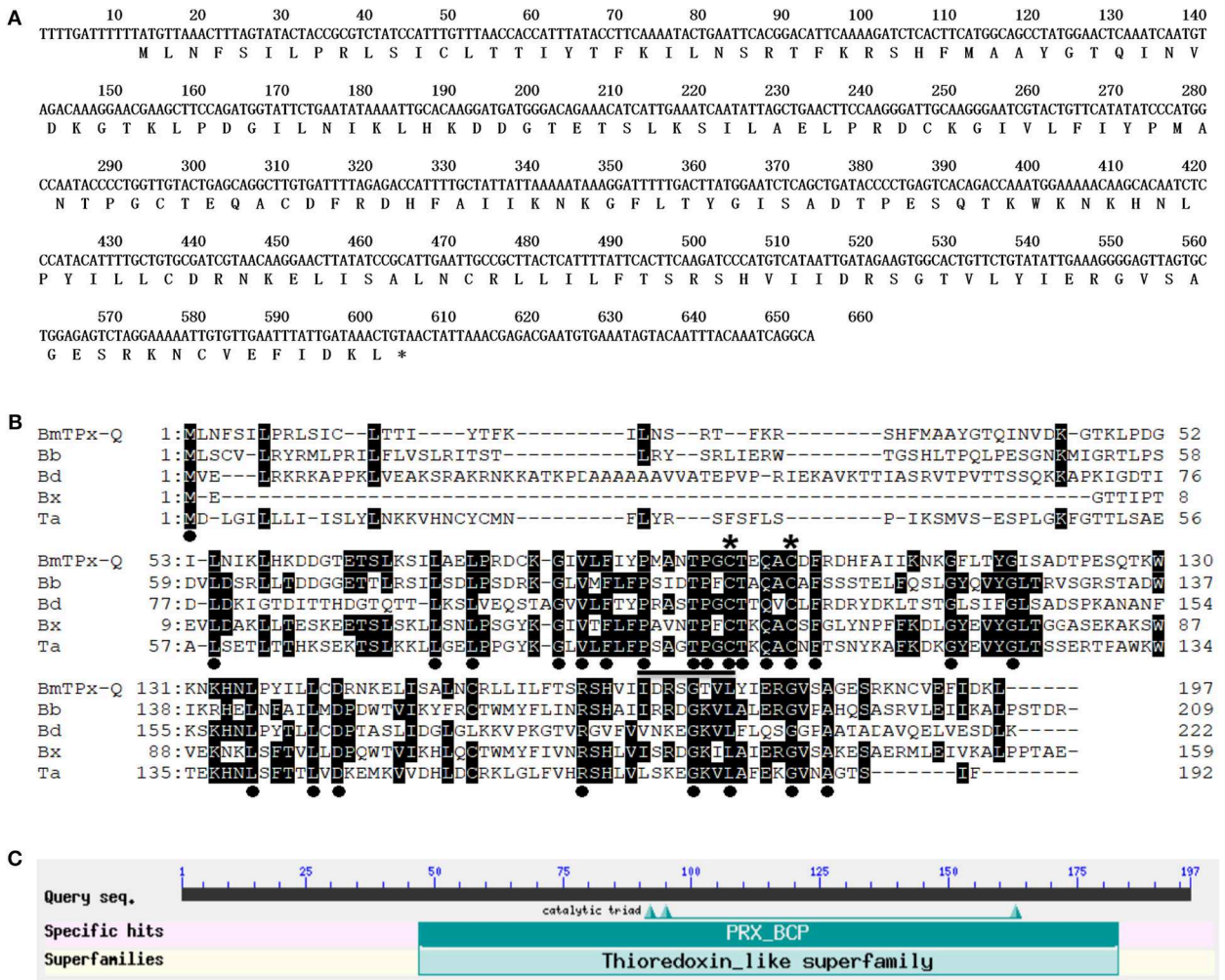

FIGURE 1 | Analysis of the sequence and primary structure of BmTPx-Q. **(A)** Nucleotide and deduced amino acid sequences of BmTPx-Q. **(B)** BmTPx-Q multiple sequence alignment analysis. *B. microti* (BmTPx-Q); *Babesia bigemina* (Bb: XP_012767998.1); *Blastomyces dermatitidis* (Bd: EEQ83458.1); *Babesia* sp. *Xinjiang* (Bx: XP_028870106.1); *Theileria annulata* (Ta: XP_954500.1). The dot indicates the identical amino acids in all sequences, an asterisk indicates the conserved cysteine residue, and the conserved PxxxTxxC-motif around the active site is underlined. **(C)** Analysis of the active domains in the BmTPx-Q amino acid sequence as identified by NCBI blast.

conserved domain PRX_BCP, 3 of 3 of the residues that compose this conserved feature (**Figure 1C**).

Expression and Purification of the Recombinant BmTPx-Q

The PCR product was cloned into the pET30a vector and the recombinant protein was successfully expressed in *E. coli* BL21 (DE3) as a his-tagged protein. The rBmTPx-Q protein was purified with Ni-NTA agarose beads and was analyzed by SDS/PAGE. As shown in **Figure 2**, the purified protein showed a single band (∼21 kDa) by 12% SDS/PAGE in the presence of DTT (reducing).

Antioxidant Activity of rBmTPx-Q

The activity was evaluated using the MFO assay (**Figure 3A**). In this assay, $FeCl_3$ and DTT generate hydroxyl radicals in the reaction mixture induced nicks in the supercoiled plasmid DNA, thereby altering the mobility of DNA during agarose electrophoresis. Both $FeCl_3$ and DTT generated nicks in the DNA in the absence of rBmTPx-Q. Thus, there was an apparent increase in DNA size (**Figure 3A**, lane 4). However, nicks in the plasmid DNA were detected after purified rBmTPx-Q was added to the reaction mixtures at 1,000, 500, and 250 μg/mL concentrations (**Figure 3A**, lanes 5–7). The results indicate rBmTPx-Q has antioxidant activity. TPx-Q proteins are thiol-based peroxidases that catalyze the reduction of H_2O_2. The ability of the rBmTPx-Q to remove H_2O_2 was evaluated utilizing a ferrithiocyanate system. The rBmTPx-Q was examined by monitoring oxidation of NADPH in the *E. coli* Trx/TrxR system in the presence of DTT. As demonstrated in **Figure 3B**, rBmTPx-Q showed peroxidase activity in the presence of the Trx system (Trx, TR, and NADPH) with a

concentration-dependent manner. These results indicate that the atypical 2-Cys TPx-Q peroxidases use thioredoxin as a reductant. The results showed a decrease at A_{340} nm in the presence of rBmTPx-Q, possibly due to the oxidation of NADPH after the addition of H_2O_2. Within 10 min, 1.5 μg of rBmTPx-Q destroyed nearly about half of 1 mM H_2O_2. This result suggested that rBmTPx-Q can be described as a thioredoxin peroxidase.

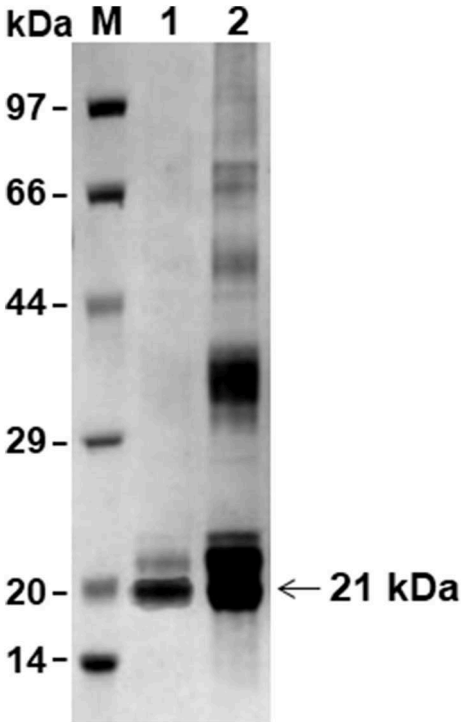

FIGURE 2 | SDS-PAGE analysis of the rBmTPx-Q-His fusion protein. Analysis of expression and purification of rBmTPx-Q by reducing SDS-PAGE (12%). Lane M: standard protein molecular weight marker; Lane 1: purified rBmTPx-Q; Lane 2: induced cell lysates. Purified rBmTPx-Q-His was ∼21 kDa and was used in subsequent antibody production and antioxidant activity assays.

Expression Analysis of BmTPx-Q in *B. microti* Post-infection

To identify the native BmTPx-Q protein in *B. microti*, western blot analysis was performed using the mouse anti-rBmTPx-Q serum. The results showed that an ∼22 kDa band was detected in the iRBC lysates 7 and 8 days post-infection (**Figure 4A**, lines 3 and 4), which revealed the predicted monomeric size, whereas no specific bands were detected in the non-infected control (**Figure 4A**, line 6). The size of the recombinant BmTPx-Q band was similar to that of the native BmTPx-Q. The results suggested that BmTPx-Q protein has high immunogenicity and can induce the host's immune system and also BmTPx-Q exists primarily in a monomeric form without the formation of intermolecular disulfide bonds. Moreover, total RNA of iRBCs from different days post-infection was used to investigate the expression profile of BmTPx-Q by qRT-PCR analysis. BmTPx-Q expression peaked twice on 4 and 8 days post-infection and declined suddenly on 5 and 9 days post-infection (**Figure 4B**). Blood smears were stained with Giemsa to assess the infection by calculating the ratio of iRBCs. *B. microti* was detected in iRBCs on the first day post-infection. The parasitemia ratio increased until 5 days post-infection, and started to decline on 6 days post-infection until reaching undetectable levels by day 10 post-infection (**Figure S1**).

Immunofluorescence Assays

A thin blood smear of *B. microti*-infected RBCs (∼20% parasitemia) was used by indirect immunofluorescence assay (IFA) with the mouse anti-rBmTPx-Q serum. The blue fluorescence indicates the nucleus of *B. microti*, whereas

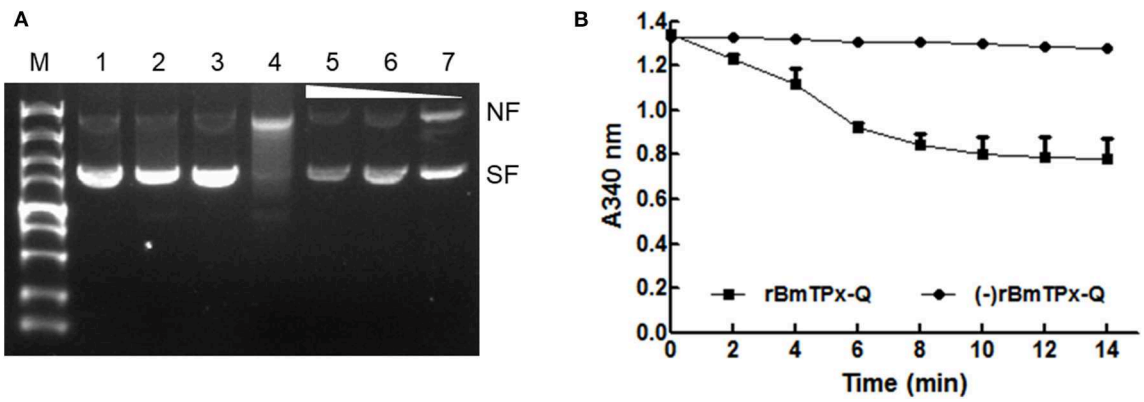

FIGURE 3 | Antioxidant activity of the BmTPx-Q protein. **(A)** The pBluescript plasmid DNA was incubated with reaction mixture including the rBmTPx-Q, and nicking of the supercoiled plasmids by MFO was evaluated on 1% agarose gels stained with ethidium bromide. M, DNA marker; 1, DNA + H_2O; 2, DNA + DTT; 3, 180 ng DNA + $FeCl_3$; 4, DNA + MFO; 5, 1,000 μg/mL rBmTPx-Q + MFO + DNA; 6, 500 μg/mL rBmTPx-Q + MFO + DNA; and 7, 250 μg/mL rBmTPx-Q + MFO + DNA. The nicked form (NF) and supercoiled form (SF) of the plasmids are indicated on the right, and the triangle shows the increasing concentration of the recombinant proteins. **(B)** Peroxidase activity of rBmTPx-Q. NADPH oxidation coupled by rBmTPx-Q (solid line) to reduction of H_2O_2 in the presence of the *E. coli* Trx/Trx R system. NADPH oxidation was monitored as the decrease in A 340 in a 200-μL reaction mixture (0.14 μM TR, 6.4 μM Trx, 0.375 mM NADPH, 500 μg/mL rBmTPx-Q, 250 μM H_2O_2, 50 mM HEPES). The dotted line indicates the absence of rBmTPx-Q in reaction mixture. Data are presented as the means of three independent experiments.

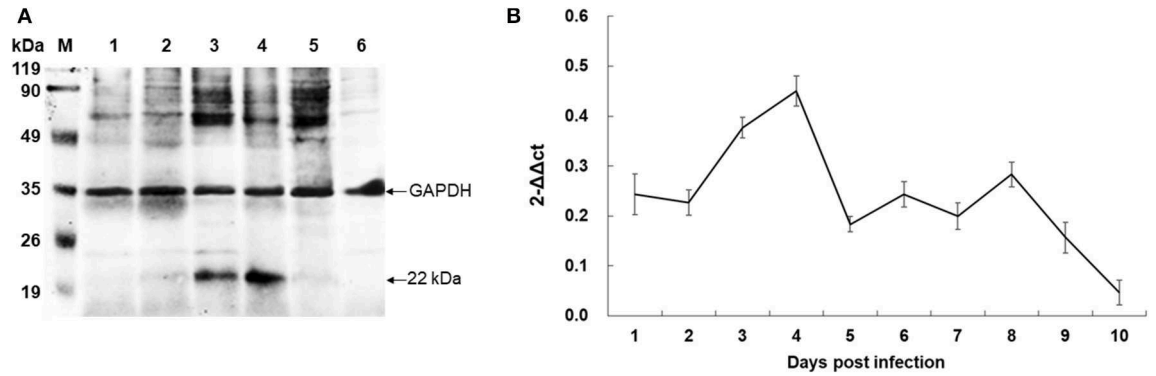

FIGURE 4 | Expression analysis of BmTPx-Q in *B. microti* post-infection. **(A)** Western blot analysis of the native BmTPx-Q. Lane M: standard protein molecular weight marker; Lanes 1–5: *B. microti* infected mouse erythrocyte lysates on 5th, 6th, 7th, 8th, and 9th day post-infection; Lane 6: uninfected mouse erythrocyte lysate; mouse anti-rBmTPx-Q serum was used as primary antibody in this Western blot analysis and revealed a band of ∼22 kDa. **(B)** Relative expression analysis of BmTPx-Q at different days post-infection. The x-axis refers to the day of infection of mouse RBCs with *B. microti*; the y-axis refers to the relative BmTPx-Q expression level in mouse RBCs on different days post-infection by using the $2^{-\Delta\Delta CT}$ mean.

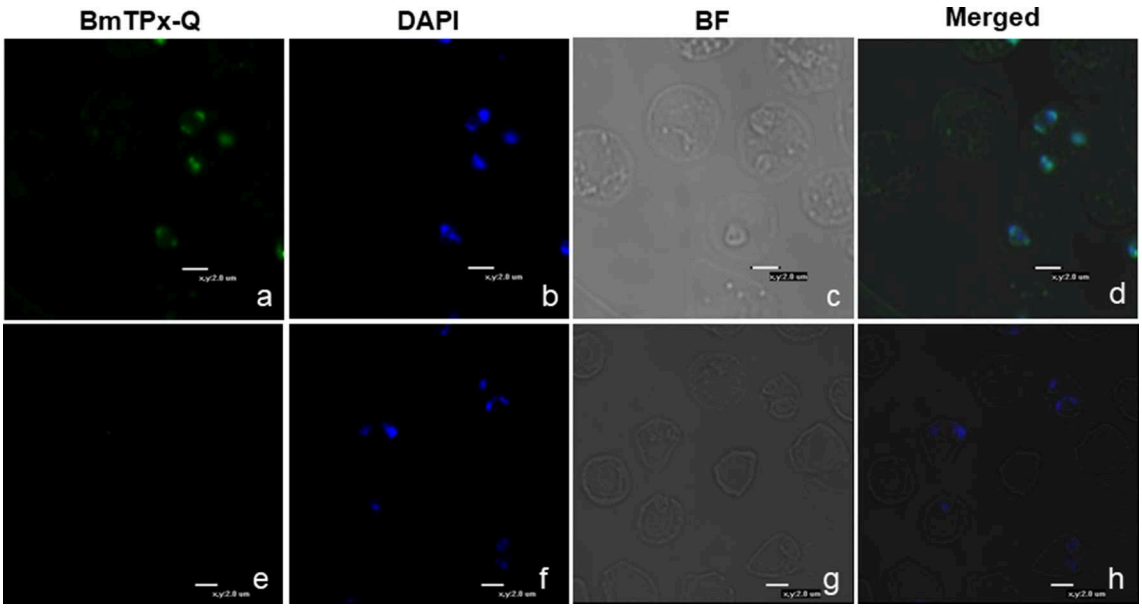

FIGURE 5 | Immunofluorescence microscopy analysis of the cellular localization of TPx-Q in *B. microti*-infected RBCs. Antiserum against BmTPx-Q **(a–d)** or normal mouse serum **(e–h)** was incubated with acetone-fixed *B. microti*-infected mice erythrocytes and stained with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody. **(a,e)** Incubation with anti-rBmTPx-Q or normal mouse serum staining; **(b,f)** DAPI staining; **(c,g)** brightfield; and **(d,h)** merged images. Scale bar indicates 2.0 μm.

the green fluorescence shows BmTPx-Q located within the nucleus of *B. microti* merozoites in iRBCs (**Figure 5a**). Additionally, co-localization of anti-BmTPx-Q signal (green) with DAPI (blue) indicates that most of BmTPx-Q is present in the cytoplasm of the parasites (**Figure 5d**, Merged). In the control sample, no green fluorescence was detected in the iRBCs which were incubated with serum collected from uninfected mice (**Figure 5e**). Therefore, our results show that BmTPx-Q is expressed in *B. microti* merozoite cytoplasm.

Transcript Analysis of BmTPx-Q After Treatment With Antiparasitic Agents

The RT-PCR was conducted using various cDNA templates isolated samples treated with different doses of Quinine monohydrochloride dehydrate, Dihydroartemisinin, or Chloroquine at 12, 24, and 36 h. As shown in **Figures 6A,B**, BmTPx-Q was expressed in three different time points, although there was no significant difference, but a decreasing trend was observed as time went by in the three different concentrations under Quinine and Dihydroartemisninin compared to the

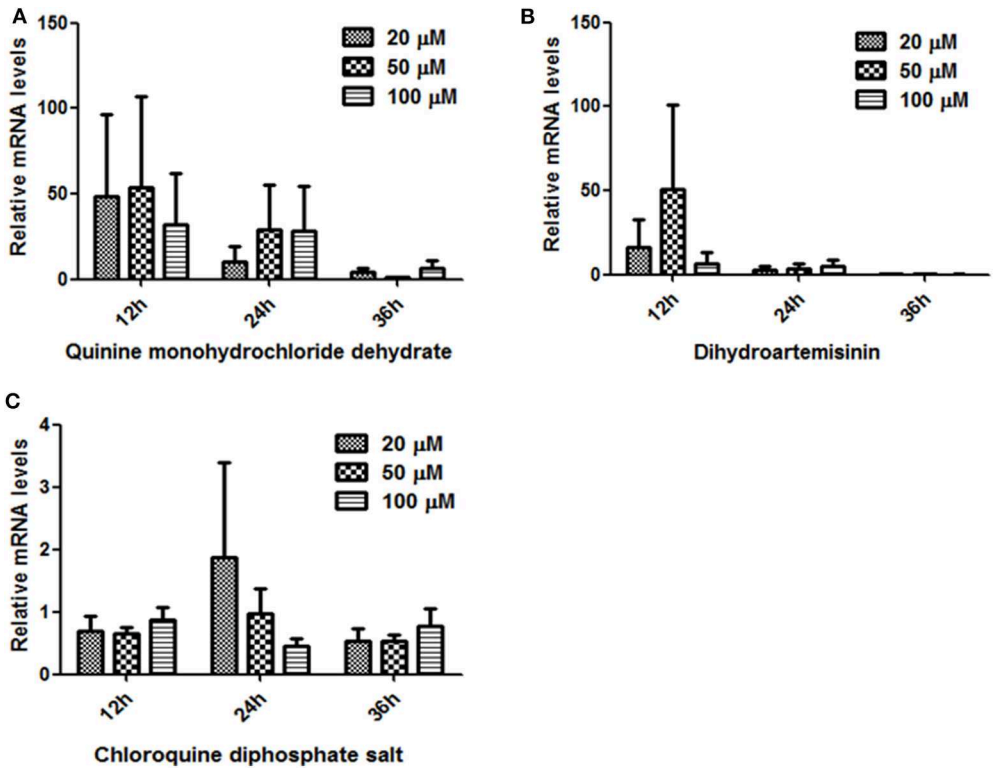

FIGURE 6 | Relative expression analysis of BmTrx-Q in iRBCs exposed to anti-parasitic agents for 12, 24, and 36 h. The iRBCs were treated with different concentrations (20, 50, 100 μM) of Quinine **(A)**, Dihydroartemisinin **(B)**, and Chloroquine **(C)**.

control group (we have subtracted the control group data when analyzing). In addition, BmTPx-Q was expressed at the highest level in 20 and 50 μM at 24 h under Chloroquine diphosphate salt (**Figure 6C**). High BmTPx-Q expression in the parasites was due to adverse environmental factors, which increases gradually to peak at 24 h before decreasing sharply in the subsequent time points. The growth status of *B. microti* after that was severely inhibited at 36 h, which was also confirmed through Giemsa-stained thin-blood films (**Figure S2**). That can explain the low expression level of BmTPx-Q in the subsequent stages, also indicating that *B. microti* might be inhibited by these three drugs. These findings suggest that BmTPx-Q might be implicated by different mechanisms in the response of *B. microti* to Quinine, Dihydroartemisninin, and Chloroquine. Although Prxs play a role in protection against oxidative damage in parasites and ensure a certain degree of defense, the effects of external factors can be irreversible.

DISCUSSION

Prxs reduce peroxides using a peroxidatic cysteine residue, and the Cys are essential for enzyme activity. In atypical 2-Cys Prxs, which are also named Type II Prxs, the position of one of two cysteine residues is not conserved (8, 46, 47). The typical 2-Cys Prxs have physiological functions as peroxidase, 1-Cys Prx and atypical 2-Cys act as monomers, whereas typical 2-Cys Prxs act as dimers (48, 49).

To better systematically understand TPxs, we previously have identified the Prxs (BmTPx-1 and BmTPx-2) from *B. microti* (36, 37). Herein, we extended these efforts to select additional members of this group. A BLAST search revealed that the BmTPx-Q has low sequence similarities with those of Prxs of host species. This may indicate that this protein may have a good prospect in terms of drug targets. As expected, like all 2-Cys-containing Prxs, BmTPx-Q possesses the PxxxTxxC-motif and beside two conserved cysteine residues Cp and Cr that are essential for peroxidase activity. The Cp is located at the N-terminal end of the protein and Cr is usually four residues or 30 amino acids away from Cp (50). In our study, BmTPx-Q as a 2-Cys Prx, Cys95 being Cp, which is essential for peroxidase activity, whereas Cys100 acts as Cr. For 2-Cys Prxs, the Cp is oxidated by a peroxide substrate to generate a Cys sulfenic acid (Cys-SOH) intermediate, which reacts with the Cr to form an monomer or dimmer (18, 51). The disulfide bond is reduced to reform the reduced Cp by an external reducing substrate of Trx/TrxR system with an atypical 2-Cys catalytic mechanism (32). Afterwards, the generated TPx-Q is ready for another catalytic cycle. Su et al. (30) indicated that the two cysteine residues are essential for enzymatic activity by mutation analysis. Furthermore, they proved that *C. glutamicum* TPx-Q catalytically

eliminates peroxides by exclusively receiving electrons from Trx/TrxR system (30). Unlike the typical 2-Cys Prx proteins, TPx-Q may exist as monomers, dimers, or a mixture (52). In this study, BmTPx-Q may exist mainly in monomeric form with an intramolecular disulfide bond. Moreover, it has been documented that the three forms of TPx-Q contain a wealth of information useful to the field (27).

To assess the enzymatic activity and generate specific antibodies against BmTPx-Q, the recombinant proteins were expressed as a his-tagged protein in *E. coli* purified by agarose beads (**Figure 2**). It is now known that Prxs are prevalent in prokaryotic and eukaryotic organisms and are essential against various oxidative stresses through catalyzing the reduction of H_2O_2 and organic hydroperoxides to keep the cellular redox balance (53). In addition, Prxs are more than just simple peroxide-eliminating enzymes. They are localized to various subcellular compartments and function as regulators of local H_2O_2 levels. The studies of transcription revealed that the Prx Q has a function in oxidant defense (54). Therefore, the antioxidant activities of rBmTPx-Q were evaluated by the MFO assay (**Figure 3A**). In MFO assay, $FeCl_3$ and DTT generate hydroxyl radicals that produced nicks in the supercoiled DNA in the reaction mixture, which could be monitored by changing the running behavior of the DNA in electrophoresis (38). Therefore, the presence of rBmTPx-Q in the reaction mixtures with 250, 500, and 1,000 μg/mL concentration prevented the damage of DNA (**Figure 3A**), suggesting the antioxidant activities of rBmTPx-Q. Data shown in **Figure 3B** demonstrated that the peroxidase activity of rBmTPx-Q acts as an antioxidant enzyme, the results were consistent that Prx as a peroxidase to reduce H_2O_2 via the parasitic Trx system in previous study (55).

Although Prxs are primarily as peroxidases, these also function as chaperones or phospholipases and are involved in redox signaling (48, 56). The chaperone or the phospholipase activity of Prxs is independent with the catalytic cysteine residues. Molecular chaperones can assist the covalent folding of proteins and prevent the protein aggregation (57). Su et al. (30) found that TPx-Q functions as molecular chaperone and peroxidase. Cho et al. (58) first reported that Prx Q of *Deinococcus radiodurans* R1 is a monomeric atypical 2-Cys Prx and has dual activity as peroxidase and a molecular chaperone. However, the molecular chaperones function of BmTPx-Q needs to be further studied.

Meanwhile, we collected the *B. microti* infected mouse erythrocyte lysates post-infection and detected the native BmTPx-Q protein in the lysate of *B. microti* using the mouse anti-rBmTPx-Q serum. Western blotting showed an ∼22 kDa band in the iRBC lysates 7 and 8 days post-infection (**Figure 4A**, lines 3 and 4). This indicates that the native BmTPx-Q was expressed in large quantities that could be detected on the 7th and 8th day after infection. Moreover, the expression of BmTPx-Q peaked at both 4 and 8 days post-infection by qRT-PCR analysis (**Figure 4B**). Based on the growth curve of parasitemia, *B. microti* was undergoing a rapid propagation process in RBCs 4 days post-infection. During the erythrocytic stages, *B. microti* are exposed to oxygen-rich environments and must secrete large amounts of antioxidants, such as Trx or Prx to act against oxidative stress and protect the parasites (23). Furthermore, the native *B. microti* Prx protects DNA from oxidative damage (23, 59). From a rational point of view, high expression levels of BmTPx-Q at 4 days post-infection helped *B. microti* to cope with the oxidative stress during the rapid propagation process. The level of BmTPx-Q expression reached a peak, whereas *B. microti* parasitemia decreased to low levels at 8 days post-infection, similar results were also observed for *B. microti* other antioxidant enzymes, such as peroxiredoxin 2, thioredoxin 2, and thioredoxin 3 in previous studies (37, 41, 42). The molecular immune mechanisms of high expression levels of these antioxidant molecules need to be further investigated.

In addition, previous research has reported that Prxs could induce protective immunity against *Leishmania* major infections and microfilaria *Brugiamalayi* infections in mice and *Fasciola hepatica* infections in goats (60). Since Prxs have potential as candidate vaccines for parasite species, the enzymatic activity may be related to the protective efficacy of these antigens. Our laboratory will evaluate the potential of this vaccine candidate in future studies.

IFA remains the most widely used method to localize proteins in organisms' intact cells. **Figure 5** shows the expression patterns of BmTPx-Q in the red blood cells. The green fluorescence surrounding merozoite nuclei indicates enzyme expression in the cytoplasm of the parasite (**Figure 5a**). Based on a previous report, 1-Cys-Prx is also upregulated in the cytoplasm at the trophozoite stage, which is the metabolically active phase of *P. falciparum* (61). Unlike thioredoxin peroxidase-1 (TPx-1) of *B. microti*, BmTPx-Q exhibits a dot-like pattern expression in the parasite. Further investigation into whether BmTPx-Q and BmTPx-1 or BmTPx-2 co-exist in the peroxidase active organelles is required.

There was no significant difference regarding the mRNA relative expression profile of BmTPx-Q in the groups treated with the antiparasitic agents, compared to the control group, but a decreasing trends were observed against time (**Figure 6**). Three drugs managed to inhibit *B. microti* growth, but there was no marked effect on BmTPx-Q expression or activity. It is necessary to find specific inhibitors or drugs able to target BmTPx-Q, which will further confirm the importance of this molecule for *B. microti* survival. Moreover, it is necessary to confirm the TPx-Q whether as an oxidative stress defensive molecule as well as drug target in other strain or species of *Babesia*.

CONCLUSIONS

We characterized a BmTPx-Q in *B. microti*, and our results suggest that TPx-Q acts as a thioredoxin-dependent monomeric peroxidase that contributes to the resistance against oxidative in *B. microti*. BmTPx-Q is a monomeric form with two Cys that form an intramolecular disulfide bond. Since BmTPx-Q has an antioxidant activity, apparently it has a crucial role in the reduction of ROS. Concerning other aspects, *Babesia* parasites have other antioxidant proteins, such as SOD and Gpx. Thus, it is necessary to study the correlations between BmTPx-Q and these antioxidant proteins. Our data on BmTPx-Q can be used to investigate the precise role and biological functions of BmTPx-Q in the parasite. These findings may be relevant to the field

of parasitological research and lead to the development of an anti-Babesiosis drug.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The Institutional Animal Care and Use Committee of the Shanghai Veterinary Research Institute (IACUC Approval Number SHVRI-mo-2017070806) approved and the Animal Ethical Committee of Shanghai Veterinary Research Institute authorized this investigation.

AUTHOR CONTRIBUTIONS

HZ and JZ conceived and designed this study. HZ and ZW performed the experiments. JH conducted the molecular analysis. JC performed the serology. YZ conducted the statistical analysis. All authors have read and approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00076/full#supplementary-material

Figure S1 | Giemsa-stained thin-blood film of the parasitemia. D0: Uninfected mouse RBCs; D1–D10: Days post-infection with *B. microti* of mouse RBCs. Scale bar indicates 5.0 μm.

Figure S2 | The growth status of *B. microti* after treatment with antiparasitic agents at 36 h. iRBCs were treated with various concentrations (20, 50, or 100 μM) of Quinine, Dihydroartemisinin, and Chloroquine at 36 h, the controls were treated with DMSO. Thin-blood film was stained with Giemsa. Scale bar indicates 10.0 μm.

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BRIEF RESEARCH REPORT
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Is Angiostrongylosis a Realistic Threat for Domestic Cats?

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Three species of *Angiostrongylus* have been found in felids thus far, i.e., *Angiostrongylus chabaudi*, *Angiostrongylus felineus* and *Angiostrongylus vasorum*. *Angiostrongylus chabaudi* lives in the right heart and pulmonary arteries of the definitive natural host, the European wildcat (*Felis silvestris*), and non-patent infections have been reported in domestic cats (*Felis catus*). *Angiostrongylus felineus*, described in the Puma yaguarondi (*Herpailurus yagouaroundi*), has never been reported in domestic felids, while recently a non-patent infection by *A. vasorum* was unequivocally described in a *F. catus*. Nonetheless, epizootiological and clinical relevance of angiostrongylosis in domestic cats are practically unknown. This study investigated whether cases of angiostrongylosis may be missed in cats living in areas enzootic for *Angiostrongylus* spp. and other metastrongyloids. Overall, 100 cats that were either positive (n.50) or negative (n.50) for metastrongyloid larvae at the Baermann's test, were examined for *Angiostrongylus* spp. with DNA-based methods and with the serological test Angio Detect™ for circulating antigen. The PCR analysis confirmed the copromicroscopy results, where 25 cats scored positive for *Aelurostrongylus abstrusus*, 16 for *Troglostrongylus brevior* and 9 for both, while no cats were positive for *Angiostrongylus*-like larvae, including *A. chabaudi*. None of the 100 sera samples scored positive at the Angio Detect™ test. These data suggest that currently feline angiostrongylosis is a minor parasitosis for domestic cats. Nevertheless, it cannot be excluded that the epizootiological drivers which have favored the spillover of *A. vasorum* and *T. brevior* from wildlife to dogs and cats, could promote the emergence of feline angiostrongylosis, with an unpredictable health impact.

Keywords: *Angiostrongylus chabaudi*, wildcat, cat, angiostrongylosis, angio detect™

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INTRODUCTION

Canine and feline cardio-pulmonary nematodes are emerging throughout Europe due to different factors (1, 2). A role in the spreading of some extra-intestinal parasites can be played by bridging infections between wildlife and domestic hosts (3, 4). This has recently led to the establishment in domestic animals of parasites which were previously considered to be affiliated only to their definitive wild hosts. As key examples, among these "new" parasites, *Angiostrongylus vasorum* causing canine angiostrongylosis, *Troglostrongylus brevior* causing feline troglostrongylosis and *Oslerus rostratus* causing feline oslerosis were previously considered typical of red foxes (*Vulpes vulpes*), European wildcats (*Felis silvestris*) and the lynx (*Lynx* spp.), respectively. These nematodes have

now a major impact on animal health, causing potentially fatal diseases in domestic canine and feline populations (4–6).

While angiostrongylosis in dogs has become a well-known disease in Europe, our knowledge on the infections caused by *Angiostrongylus* spp. in felines is very poor. *Angiostrongylus chabaudi* is a metastrongyloid nematode that was first described in wildcats from Central Italy in 1957 (7), and that has not been reported again until few years ago, when it was found in a domestic cat (*Felis catus*) from Sardinia (Italy) as a non-patent infection (8). Then, this parasite has been documented in another non-patently infected domestic cat from Central Italy (9) and in wildcats from Germany (10), Italy (11), Greece (12, 13), Romania (14), Bulgaria (15), Bosnia and Herzegovina (16). Recently, the European wildcat has been shown to be the definitive host of *A. chabaudi*, with a demonstration of patent infections and presence of first stage larvae (L1) in the feces for the first time (12). The clinical importance of angiostrongylosis caused by *A. chabaudi* in domestic cats is virtually unknown, although pathological lesions (e.g., granulomatous pneumonia, parenchymal hemorrhagies, alveolar emphysema, subendothelial proliferation and oedema, thrombosis and hyperplasia/hypertrophy of the pulmonary arteries) have been reported in both wild and domestic cats (9, 11, 12, 16). With regard to other *Angiostrongylus* species, the ability of *A. vasorum* to infect felid hosts was shown under experimental conditions (17, 18), and recently, a case of natural infection has been described in a domestic cat from Switzerland (19). However, to date patent infections caused by *A. vasorum* in *F. catus* have never been described neither in experimental nor in natural conditions. Few years ago, *Angiostrongylus felineus* has been described for the first time in the Puma yaguarondi (*Herpailurus yagouaroundi*) from Brazil (20), but at present no cases of infection have been documented in domestic cats.

The diagnosis of feline cardio-respiratory parasitic infections is currently based on the morphometric and morphologic identification of L1 shed in faces using the Baermann test (21). A key limitation in diagnosing possible angiostrongylosis in domestic cats is due to the fact that at the moment there is no any demonstrations of patent infections. This absence of data could have been caused by missed diagnosis due to a certain similarity of microscopic features of *Angiostrongylus* with those of other metastrongyloids more commonly detected in fecal samples of cats (i.e., *Aelurostrongylus abstrusus* and *T. brevior*) (12).

DNA-based assays are powerful to identify metastrongyloid L1s in the feces of cats (22), thus would have the potential to reveal missed diagnosis at the microscopic analysis. The rapid serological test Angio Detect™ (IDEXX Laboratories Inc.) is able to detect circulating antigens produced by *A. vasorum* infecting dogs (23). Interestingly, this test has recently proved useful for the detection of other *Angiostrongylus* species, i.e., *A. chabaudi* in European wildcats and *Angiostrongylus daskalovi* in badgers (24). Being able to detect *Angiostrongylus* spp. circulating antigens, this test could also have a potential to diagnose feline angiostrongylosis.

The present study has aimed at investigating for the first time whether angiostrongylosis is a potentially missed, neglected or underestimated disease in domestic cats by using DNA-based and serological tests.

MATERIALS AND METHODS

One-hundred domestic cats from regions of Italy and Greece enzootic for *Angiostrongylus* spp. and major feline cardio-respiratory nematodes (2, 9, 11–13) were examined for the presence of metastrongyloid larvae. Animals were selected as a convenient dataset, as they were referred for routine clinical examinations and/or presence of compatible clinical signs. All animals were not sampled purposely for the present study and were not subjected to unnecessary suffering. Only the surplus material deriving from blood samplings expressly requested by the owner and/or indicated by the examining veterinarian for other diagnostic purposes was used in this study. Healthy animals were sampled providing that they were from enzootic areas and had a free-roaming lifestyle. From one to three consecutive fecal samples were examined for each cat by a Baermann's examination (2). Cats were divided in two groups, i.e., cats that scored either positive (G1) or negative (G2) for metastrongyloid larvae at the fecal test. All larvae collected at the Baermann test were examined using morphological and morphometric keys (12, 21). All Baermann's sediments were subjected to a multiplex-PCR as previously described (22) that is able to simultaneously detect *A. chabaudi, Ae. abstrusus,* and *T. brevior*. An individual blood sample was obtained for each cat with the owner's consent. Samples were collected for veterinary hematological examinations (e.g., routine or other diagnostic tests). Samples were subjected to centrifugation to obtain the serum. All sera samples were examined using the Angio Detect™ test (IDEXX Laboratories, Westbrook, Maine, USA), according to the manufacturer's instructions. More details are reported in Schnyder et al. (23).

RESULTS AND DISCUSSION

Out of the 50 Baermann-positive cats (G1), 25 were microscopically positive for *Ae. abstrusus*, 16 for *T. brevior* and nine were positive for both nematodes. None of the samples scored positive for *Angiostrongylus*-like larvae nor for *O. rostratus* (the latter was not surprising as *O. rostratus* larvae usually do not migrate in water). PCR results confirmed the microscopic identification of the L1s retrieved in the Baermann sediment and there were no PCR-positive for samples negative at the copromicroscopy. All sera samples scored negative for circulating *Angiostrongylus* antigen at the Angio Detect™ (**Table 1**).

TABLE 1 | Results of the Baermann examination confirmed by molecular analysis (*) and of the serological Angio Detect™ test performed in the present study.

	Italy n/tot (%)	Greece n/tot (%)	Total n/tot (%)
*Aelurostrongylus abstrusus**	23/64 (35.9)	2/36 (5.5)	25/100 (25)
*Troglostrongylus brevior**	15/64 (23.4)	1/36 (2.7)	16/100 (16)
Aelurostrongylus abstrusus + *Troglostrongylus brevior**	8/64 (12.5)	1/36 (2.7)	9/100 (9)
*Angiostrongylus chabaudi**	0/64 (0)	0/36 (0)	0/100 (0)
Angio Detect™	0/64 (0)	0/64 (0)	0/100 (0)

Although preliminary, this study suggests that cats living in areas where *Angiostrongylus* is present even with high infection rates (2, 11–13), are realistically at a null or minor risk of acquiring angiostrongylosis. The absence of *A. chabaudi* L1s in potentially infected cats could be due to the inability of this metastrongyloid to reach the adult stage in domestic hosts (8, 9). To date, indeed, only two cases of *A. chabaudi* infection have been described in domestic cats, where necropsy examinations showed immature nematodes in the pulmonary arteries without evidence of L1 in feces (8, 9). In a recent survey, the Angio Detect™ test showed a 97.1% sensitivity and a 98.9% specificity in detecting *A. vasorum* infection in dogs (25) and another study carried out on wildcats and badgers positive for *A. chabaudi* and *A. daskalovi,* respectively, showed 100% correlation between necropsy results, confirmed by molecular assays, and serologic positivity to the Angio Detect™ test (24). The sero-negativity of G1 cats suggests a lack of cross-reactivity between the different lungworm species infecting cats. In fact, it seems that the Angio Detect™ test can cross-react exclusively between species of the genus *Angiostrongylus* (24). This might be also explained by their colonization of blood vessels and thus the direct availability of circulating antigen in blood samples, as it is the case with the heartworm, *Dirofilaria immitis*. It can be assumed that this rapid assay has an elevated sensitivity, although not 100% (25), either in wildcats or in domestic cats, because its reliability does not seem to be influenced by the tested species, having been used successfully in non-canine hosts (24). Furthermore, the Angio Detect™ is able to detect the infection also in absence of *Angiostrongylus* spp. L1 in feces, as long as the parasites have reached adulthood (25). Therefore, it is unlikely that the cats of the present study were false negative, having been tested with three different diagnostic methods and the serologic negativity is most probably due to a real absence of *A. chabaudi*, *A. vasorum,* or other *Angiostrongylus* species. In fact, cats of G1, shedding larvae, would have scored positive at least at one of the tests performed in the present study. This is supported by the fact that all cats of both G1 and G2 scored negative for *Angiostrongylus* spp., other than the Angio Detect™ test, also at the molecular analysis for *A. chabaudi*, which seems to be the major *Angiostrongylus* infecting felines. Nonetheless, it should be considered that the antigens detectable with this rapid assay are produced by adult *Angiostrongylus* worms (23, 26) and that *A. chabaudi* and *A. vasorum* are most probably unable to reach adulthood producing detectable antigens in domestic cats (8, 9, 19). Indeed, a nematode found in a pulmonary artery of a domestic cat, whose section size and content were suggestive of an adult stage, was identified as *A. vasorum* at PCR analysis despite the cat scored negative at the Angio Detect™ test (19). Such negativity could be due to different reasons, including a <100% sensitivity of the test (25) or to the fact that the first antigens are detectable 9 weeks post-infection (23). As development of *A. vasorum* to adult may occur as early as 28 days post-infection (27) false negative results might occur with this test even in presence of adult nematodes. The herein examined cats could have scored as false negatives only in a similar scenario, where *A. vasorum* infection confirmation could be possible only with necropsy and molecular analysis on the nematodes retrieved in the arteries. Although this possibility cannot be ultimately excluded, these results indicate that angiostrongylosis, at present, is still an infection of minor importance for domestic cats. However, possible bridging infections by *Angiostrongylus* in future, especially *A. chabaudi*, between wildcats and domestic cats cannot be ruled out. In fact, different factors are influencing domestic and wild fauna interactions, e.g., urbanization and reduction of wildlife habitats (2). Thus, spill-over events similar to those that have likely occurred for *A. vasorum* in dogs and *T. brevior* in cats (4, 6) may indicate that similar eco-epizootiological modifications could have the potential to influence biology and epizootiology of related parasites belonging to the genus *Angiostrongylus*. A possible spreading of feline angiostrongylosis could have a severe clinical relevance in domestic cats, as happened for canine angiostrongylosis. Angiostrongylosis by *A. vasorum* in foxes, i.e., the natural reservoir, appears to be milder that in dogs, with a moderate impact on the general health status, and with pathological lesions suggestive of a mainly chronic course (28–30), while canine angiostrongylosis is a well-known disease with a possible fatal outcome (6).

In conclusion, domestic cats seem to be at low risk of angiostrongylosis, even in areas where wildcats and domestic cats live in sympatry and where different species of *Angiostrongylus* occur.

It is interesting to note also that the worldwide spread land snail *Cornu aspersum* may act as intermediate host of *A. chabaudi*, *A. vasorum,* and *Ae. abstrusus* (31–33). Despite *A. vasorum* and *Ae. abstrusus* may be present in the same geographic areas, possibly developing in *C. aspersum*, this is not the case of *A. chabaudi*. This could be due to the very low presence of this nematode in its reservoirs, that is also reflected by a low presence of infective stages in mollusks and paratenic hosts.

As future outbreaks of feline angiostrongylosis with an unforeseeable impact on feline health should be taken into account, constant epizootiological surveillance appears to be crucial. The use of Angio Detect™ is promising for its use in feline medicine under both clinical and epizootiological standpoints although the diagnostic efficiency of this test in cats that have non-patent infections remains to be understood. As the identification of *Angiostrongylus* L1 in cats can be impaired by the similarity in morphologic and morphometric features with other metastrongyloids that can be usually found at the Baermann's examination (12, 21), the use of combined diagnostic approaches including copromicroscopy, DNA-based assays and rapid serological kits may minimize the chances of false negative results and is herein encouraged in cats that could be at risk of infection by *Angiostrongylus*.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical approval for this study was not required according to national/local legislation because no cats were sampled or subjected to unnecessary suffering. The animals included were not bled for this study as only the surplus material deriving from blood samplings expressly requested by the owner and/or indicated by the examining veterinarian for other diagnostic purposes was used in this study.

AUTHOR CONTRIBUTIONS

ADC, SM, and DT participated in study activities and in drafting and revising the manuscript. MC, GS, FV, FM, AD, RD'A, and EP participated in the field and laboratory work. NP participated in the study design and in interpreting the serological results. All authors have participated in critically revising the manuscript.

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REVIEW
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Habronematidosis in Equids: Current Status, Advances, Future Challenges

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Over the past few decades, among equine parasitoses caused by gastrointestinal nematodes, habronematidosis has been discontinuously studied worldwide. Habronematidosis is a parasitic disease distributed all over the world. It is caused by *Habronema microstoma*, *Habronema muscae,* and *Draschia megastoma* (Spirurida, Habronematidae), and it is maintained in the environment by muscid flies which act as intermediate hosts. At larval and adult stages these species live in the stomach of domestic and wild equids. However, the larvae can also be found on the skin, causing lesions known as "summer sores", and occasionally on other body areas, such as ocular and genital mucosa (muco-cutaneous habronematidosis) and lung, liver, brain parenchyma. Depending on the parasite's developmental stage and localization site, clinical signs vary from mild to severe. Habronematidosis is responsible for significant economic losses, mostly when sport horses are affected, because their performances are impaired and the infection can be unaesthetic. We used three on-line databases for searching the articles on habronematidosis according to the selected inclusion criteria; a total of 250 contributions, published between 1911 and 2020 were analyzed. This review summarizes the key features of pathogenesis, epizootiology, diagnosis, treatment, and control of habronematidosis, and highlights the current knownledge about its geographical distribution and spread. Anthelmintic drugs are the most widely-used tools against habronematidosis; given the known risk of anthelmintic resistance in some nematodes affecting horses, this aspect should also be explored for habronematidosis. Dedicated research is essential to fill gaps of knowledge and increase the understanding of habronematidosis to maximize equine health, reduce economic losses and sanitary impact associated with this parasitic infection.

Keywords: habronematidosis, epizootiology, clinical signs, diagnosis, control

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INTRODUCTION

The fascinating history of habronemiasis—from now on called habronematidosis—dates back to the second decade of 1900, when in an interesting Special Article published on *Science*, Ransom (1), a bright zoologist of the Bureau of Animal Industry, in Washington DC, states: "*Fifty years ago, from Bombay, India, the late H. J. Carter reported the discovery of nematodes parasitic in the house fly, giving them the name of* Filaria muscae."

This means that the fortune of this parasite derived from its fortuitous discovery not in the animal victim but in its intermediate host. In fact, when in the summer of 1911, "*a series of stages in the development of the parasite was obtained by examination of various stages of the fly from larva to imago*" (…), the hypothesis that "Habronema muscae *is the larval stage of a nematode parasitic during its adult stage in (….) the stomach of the horse*" was postulated. This was confirmed soon later when in a few horses "*examined shortly after death*" (…), not only "*a few adult nematodes were found*" but also "*a complete series of stages in the development and growth of a single species of nematode from larva to adult*".

Indeed, the knowledge of the entire life-cycle of *Habronema* was described thanks to the results of those old and pioneering investigations.

Since that intense period of studies, habronematidosis has been intermittently studied with none or a few original and on the field studies up to the '80s. In this decade, the knowledge of this parasitosis had a significant impulse following the discovery (in the late-1970s) of the innovative drug ivermectin (2), introduced commercially in 1981. More often, these studies on habronematidosis coincided with specific investigations on the gastrointestinal nematodofauna (from prevalence to pathological aspects) in slaughtered horses finalized later on to the control of these parasites. Afterward, only a few studies dealt specifically with habronematidosis until the attention to sport horses highlighted the importance of the "summer sores" in these valuable animals and the need of controlling gastric forms of these spirurids in the definitive hosts. Thereafter, the advent of molecular tools opened new interesting scenarios for the comprehension of this widespread—but still not completely understood—intriguing parasitic disease.

Here, a broad review of habronematidosis, covering all aspects (etiology, epizootiology, clinics, pathology, diagnosis, prophylaxis, and therapy) were undertaken. In addition, key weaknesses and knowledge gaps were identified and key suggestions for future research were provided. Thus, we scrutinized 250 articles/books published between May 1911, and April 2020, with no language restrictions[1].

[1]Search strategy and selection criteria: The articles cited in this Review were searches by the PubMed, Scopus, and Google Scholar databases (up to April 10, 2020) and carried out using the terms "habronem*" AND ("taxonomy" OR "nomenclature"); "habronem*" AND (life-cycle" OR "transmission" OR "host"); "habronem*" AND ("histo*" OR "clinical manifestation" OR "syndrome"); "habronem *" AND ("epidemiology" OR "epizootiology" OR "prevalence"); "habronem *" AND ("horse" OR "donkey" OR "equid"); "habronem*" AND ("season"); "habronem*" AND ("detection" OR "diagnosis" OR "microscopy"); "habronem*" AND ("treatment" OR "chemotherapy"); "habronem*" AND ("immunol*"); "habronem*" AND ("prevention" OR "control"; "habronem*" AND ("molecular" OR "genom*"). The same searches were conducted using the term "draschi*" and "habronematid". A total of 250 publications were identified; we screened the titles and abstracts and identified articles with relevant content and context. The full texts of these articles were read to verify their relevance to the present topics in this Review.

ETIOLOGY AND LIFE CYCLE

Among the 12 species of *Habronema* listed as parasites of mammals (3), *Habronema microstoma* (syn. *Habronema majus*[2]), *Habronema muscae*, and *Draschia megastoma* (former *Habronema megastoma*) (Spirurida, Habronematidae) are the only ones detected in domestic (horses, donkeys, mules) and wild equids (zebras).

Habronema microstoma is a whitish worm (female and male, 15–35 and 9–22 mm of length, respectively), narrowed slightly at the anterior end, with a single lateral ala. The buccal vestibule is greatly thickened and has two tridentate teeth (5). The pharynx is cylindrical and provided with a dorsal and a ventral tooth, called "pharynx teeth" (5, 6).

Habronema muscae, closely resembles *H. microstoma*; the differences concern the color as adult (yellow pale or orange) and the pharynx, which is not provided with teeth.

Draschia megastoma adults (7–13 mm long) are white and their head is separated from the rest of their body by a visible constriction. The pharynx is funnel-like, with two separated lateral vales; no teeth are present (7).

The adults of all three species (*Habronema muscae*, *H. microstoma* and *D. megastoma*) live in the stomach wall of the gastric fundus and pyloric valve or freely on the mucosal surface of the *margo plicatus* (8). After mating, females release eggs (40–80 μ × 10–20 μ in size—with *H. muscae* reaching the biggest size), elongate in *Habronema* and cylindrical in *Draschia*, containing larvae, which may either hatch during intestinal transit or in the environment after release via feces. The first stage larvae (L1) are motile and show a positive hydrotropism and thermotropism; they can live as long as 7 days under suitable environmental conditions. Eggs and/or larvae are then ingested by dung-inhabiting muscid larvae. *Musca domestica* and *Stomoxys calcitrans* are the main vectors of *H. muscae* and *H. microstoma*, respectively. The larvae and the insect develop synchronously. In fact, *H. microstoma* and *H. muscae* develop further at about a similar time as the fly *imago* emerges from the *puparium* (1, 9).

The larval development of *H. muscae* in *M. domestica* in laboratory conditions has been nicely described by Amado et al. (10). Three to 5 days post infection, *Habronema* L1 were found free in the hemocoel and in fat cells of muscid L3. From 4th to 7th days p.i. two morphotypes of L2, robust and elongate, respectively, can be simultaneously present. The first is located in intracellular fat cell-like structures whereas the elongate form is located into capsules formed by syncytial tissue; the robust type was recovered from fly larvae post-feeding whereas both morphotypes from cryptocephalic pupae, pupae and pharate adults. *Habronema* L3 were found in thin and elastic capsules inside the mature pupae and adults, fixed to different fly organs,

[2]Most authors consider *Habronema majus* and *Habronema microstoma* as the same species. Recent morphological findings related to the arrangement of the caudal papillae in the male and molecular (in *ITS2* and in *cox1*) interspecific differences between *H. majus* and *H. microstoma* from donkeys indicate that they could be separate species (4).

including middle and final intestine. From 48 h post-emergence L3 reach the fly head (10).

Stimulated by the warmth, *Habronema* L3 are deposited by the flies around the animals' lips; horses swallow them and the larvae develop into adults in the animals' stomach, causing the gastric form. When the larvae are deposited on other cutaneous sites (cutaneous form) or eyes, nostrils, genital mucosa (muco-cutaneous form), or when, as rarely occur, they reach the lungs (pulmonary form), liver, brain (erratic form) (11), they do not achieve sexual maturity.

EPIZOOTIOLOGY

Habronematidosis is distributed worldwide mostly in tropical and subtropical areas, but it is also prevalent (enzootic) in temperate regions, including the Mediterranean countries (12). Prevalence differs significantly among countries and data comparison was difficult due to the limitations and large differences in the study designs. Taking into account only medium/large-scale epizoological investigations, gastric habronematidosis has been reported in Europe affecting 1.1% of horses in Sweden (13), 4.3% in The Netherlands (14), 8.5% in France (15), 17% in Belgium (16), roughly 20% in Poland (17), 33% in Germany (18). In North America the prevalence ranges from 11 to 62% (19), whereas in Australia it reaches peaks of 72% (20, 21). In Africa there is the highest infection prevalence: 62–100% of donkeys and/or horses were found positive (22–27).

Up to 2,000 individual parasites (mean 500) have been counted in a single animal stomach (28); however, in some areas (i.e., Morocco), up to 4,000 individuals have been detected (23).

Although cutaneous or muco-cutaneous habronematidosis are described, especially in temperate regions (29), the prevalence of these forms is lacking, mainly because of clinical diagnosis limitations. The description of these cases is often limited to single cases, for instance in UK (30, 31), Belgium (32), and Italy (12). An increase of (peri)ocular habronematidosis has been recently suspected in the Netherlands (33).

Although the responsible for the infestation is often unidentified, both in gastric and cutaneous forms, when identifications occur, regardless the forms, *H. muscae* is the most detected species. *Draschia megastoma*—originally described from horses in Germany (34)—is currently considered a rare parasite (17, 18, 35–37). It is a frequent species in the USA where the percentage of positive animals ranges from 24 to 62% (19, 28, 38) or in Australia where the infection rate varies from 39 to 41% (20, 21).

For both gastric and cutaneous habronematidosis, the infection does not seem to be age dependent (39).

Although several fly genera of Diptera (Muscidae) (*Musca*, *Fannia*, *Sarcophaga*, *Haematobia*, *S. calcitrans*) have been incriminated as possible vectors of habronematidosis in field conditions, only *S. calcitrans* and *M. domestica* have been proven to transmit *H. microstoma* and *H. muscae*, respectively (40). These two species are the most closely associated with the environments where horses are kept. Larval stages of both species are dung-inhabitants; adults of *M. domestica* (secretophagous) feed on eyes, nose and mouth of the host, whereas *S. calcitrans* are blood-feeding, and attack the animals mostly on their legs and flanks.

Interestingly, it has been recently shown that (i) longer is the exposition of *M. domestica* to *H. muscae*, higher is the average larval burden of *H. muscae* in the emerged flies; (ii) the proportion of insect larvae that develop into adults is lower in infected groups; (iii) in infected groups pupae are smaller and lighter. Whether this is attributable to the destruction of adipose cells in the maggots by *Habronema* larvae or not, requires a more in-depth investigation (41).

The seasonality of the intermediate hosts influences the seasonal trend of habronematidosis. In temperate climates, the infection reaches its peak in summer; in tropical areas the spread of *H. microstoma* reaches high levels in January and in July-September, while *H. muscae* especially in January-March (23); it is therefore conceivable that, at least in some regions, the two *Habronema* species have "preferential" species of flies as intermediate hosts.

It is interesting to point out that, whilst the role of *M. domestica* as a vector of *H. microstoma* remains to be better investigated (40, 42), the host-parasite association between *M. domestica* and *H. muscae* appears more biologically and developmentally settled. This seems to be related to the ability of *M. domestica* to stabilize *H. muscae*; in fact, although there is an inverse relation between the intensity of infection by *H. muscae* and the longevity of *M. domestica*, a low level of infestation does not interfere with the dipterous reproduction and consequently guarantees the maintenance of habronematidosis (43). This aspect confirms the adaptation process related to coevolutive processes. Furthermore, the presence of ultrastructural "anatomical devices" on *H. muscae* infective L3 seems to help them in the rupture of the muscoid proboscis and in the movements to reach the horse (10). At the same time, it cannot be excluded that the strong ability of *M. domestica* in transmitting *H. muscae* may be related to the high number of infected houseflies in horse farms together with a high prevalence and mean intensity of *H. muscae* infection in horses (10).

CLINICAL SIGNS AND HISTOPATHOLOGICAL ASPECTS

Clinical signs related to *H. microstoma*, *H. muscae,* and *D. megastoma* infection depend on the parasite's stage of development and localization. Adults have a double effect on the host: a mechanical-irritative and a toxic effect, caused by the metabolites they produce.

At least four clinical forms of habronematidosis are known, according to the localization of the nematodes.

In the *gastric habronematidosis*, *Habronema,* and/or *Draschia* are confined at the level of gastric mucosa glands and responsible of different degrees of atrophy, mechanical irritation of the stomach, secretory and functional disorders; clinical signs can range from no signs to anorexia/dysorexia, digestive disorders, diarrhea, gradual weight loss; also they may predispose horses to

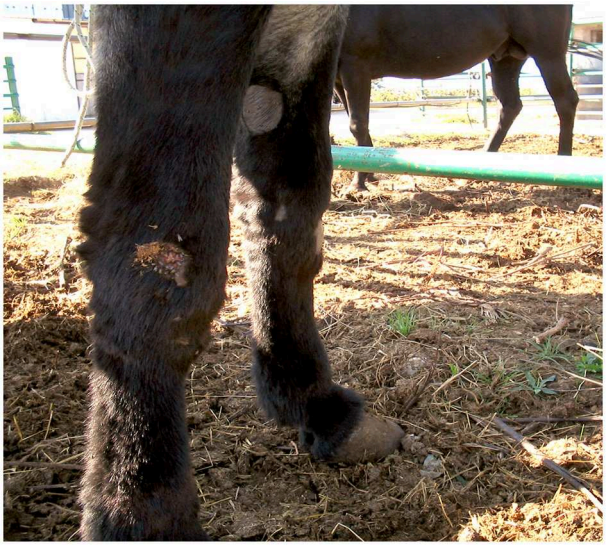

FIGURE 1 | Cutaneous habronemosis in a donkey (original, D. Traversa).

ulcers and postprandial colics (44, 45). Adults of *D. megastoma* create large swellings, which may hamper the peristalsis of the stomach, or impede the pyloric opening. This nematode occasionally causes acute hemorrhages or damage of the stomach wall leading to acute peritonitis and even death (46, 47). There are often congested and hemorrhagic ulcer-like areas, which can be isolated or confluent, especially when *H. microstoma* is present. *D. megastoma* causes granulomatous lesions with central necrosis, cellular debris and eosinophilic infiltration. When *H. microstoma* and/or *H. muscae* is present, an abundant secretion envelops the parasites. A close agreement between the number of infected horses by *D. megastoma* and the presence of lesions have been also noticed (19).

In the stomach of affected donkeys, at necropsy hyperaemia, erosions and ulcers, oedema, together with parasitic lesions are visible. Ulceration of the non-glandular gastric regions is more prominent than the glandular regions (48). Histologically, hyperkeratosis, acanthosis, vacuolar degeneration of squamous cells, erosions, ulcerations, hyperfunction of mucus glands have been described in donkeys (48).

The *cutaneous habronematidosis* is the most severe form, and lesions are known as "summer sores" (**Figure 1**). Wounds tend to disappear spontaneously in the cold months but re-appear when the environmental temperature rises again months later. It is still unclear whether in winter the larvae remain in the lesions in a dormant state, and reactivate later in the following warm season or not (49). L3 are deposited on the wounds by flies and the spine at the larval posterior end is responsible for the injury and for the local hypersensitivity reaction. Chest, fetlocks and the inner side of the legs are the most affected body areas.

Clinical signs range depending on the origin/time of the lesion. Skin lesions may be single or multiple and are proliferative, exuberant and granulomatous, frequently bloody, itchy, and ulcerated, and contain necrotic, caseous or calcified granules (12, 50, 51).

If the lesion originates from a pre-existing lesion (as is typically the case), the wound develops into: dry, wet and edematous forms. The dry lesion is a generally circular alopecic area covered by grayish scales. The wet lesion is associated with moderate discharge and hair agglutination, whilst the edematous lesion is hairy and does not have a regular shape (3–5 cm in diameter); it is characterized by oedema and tiny nodules.

Lesions may heal (**Figure 2**) or recurrent lesions may evolve into non-healing granulomatous cancer-like masses; these may attract more flies, leading to a super-infection (9, 51, 52). Histologically, the wounds are infiltrated with eosinophils, macrophages, lymphocytes and a few plasmacells. In peripheral areas, an abundance of vascular and fibro-connective tissue can be observed, with masses of eosinophils in coagulation necrosis. Sections of nematodes can be also detected (53) (**Figure 3**).

In the *muco-cutaneous habronematidosis* conjunctiva, medial canthus, nasolacrimal ducts, or commissure of the lips or urethral process, glans, prepuce, vaginal fornix are involved. When larvae are released in the eyes or on the periocular tissue, typically in the medial canthus, infected animals present marked conjunctivitis, blepharitis, dermatitis with photophobia and lacrimation (6, 32, 54).

This form is only apparently uncommon; five cases of (peri)ocular habronematidosis have been recently published in the Netherlands (33) but we speculate that many other cases might have not been published. Affected horses may show profuse mucopurulent discharge, and from moderate to evident blepharospasm and/or epiphora. Some may suffer of ectropion and chemosis. Gross lesions are ulcerative and granulomatous with so-called sulfur-like granules within and around the lesion (from 5 mm to 1.5 cm in diameter up to 25 × 10 cm) that appear on the palpebral conjunctiva of the medial canthus (33, 55). The histological examination shows a marked infiltration of multifocal to coalescing eosinophilic granulomas, and a nucleus of eosinophilic necrotic debris together with many degenerate eosinophils delimited by epithelioid macrophages with few lymphocytes and plasma cells in the adjacent tissue, with occasionally multifocal moderate to large clusters of coccoid bacteria in most of the affected animals (33). When the prepuce, urethral process, vaginal fornix are affected, animals show dysuria and frequent urination due to the presence of different degrees of fibrosis (56, 57). Histological exam of the mucous membranes reveals granulation tissue, infiltrated by eosinophils and affected by collagenolytic phenomena (54).

The pathogenesis of the *pulmonary habronematidosis* is unclear, and how larvae reach the lungs is not fully understood (44, 46, 58). Larvae on skin wounds may move to the lungs via the bloodstream, or larvae released in the nostrils or mouth mucosa can reach the lungs via the trachea (59). In any case, the parasites damage the peribronchial tissue causing large nodules—0.2–2 cm of diameter—which contain larvae or residues of larvae (42).

Erratic forms are described for *D. megastoma*; larvae belonging to this species can reach the brain and form small nodules (60).

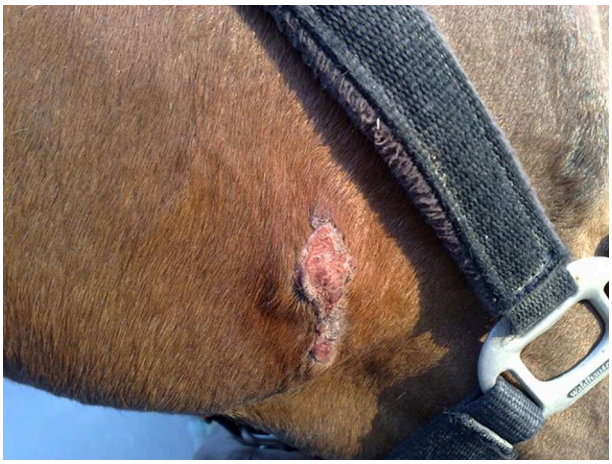

FIGURE 2 | Healing process of a summer sore (original, A. Giangaspero).

DIAGNOSIS

Gastric habronematidosis cannot be easily diagnosed, because the unspecific clinical signs, that characterize this form, may easily be confused with other diseases. The detection of larvated eggs by flotation or—although less successfully—of larvae using the Baermann technique or coproculture has been widely performed. The xenodiagnosis is considered the gold standard even though it is challenging and time-consuming; in alternative, a modified Mertiolate-Formaldehyd-Concentration (MFC) technique has been proposed for suspected gastric forms by *H. muscae* (61). All these traditional techniques have a very low sensitivity, even if the parasitic burden is high.

Another diagnostic approach is gastric lavage combined with microscopic examination of the sedimented washings, but this method is invasive, dangerous and laborious; in addition, the patient has to be necessarily anesthetized and restrained.

Differental diagnosis of cutaneous and muco-cutaneous forms is also challenging, in fact, the clinical signs, the granulomatous lesions in particular, may overlap those of other diseases, i.e., botryomycosis, pythiosis, phycomycosis, onchocercosis, equine sarcoid, and squamous cell carcinoma (54, 62–65). Diagnosis may be even more challenging when coexistence of sarcoid and habronemosis occur (66). The surface of the lesion must be scratched in different areas and, in order to detect the larvae, the collected tissue has to be digested for 12–18 hours at 37°C in an acid pepsin solution (50). However, the larvae tend to be few, and might be digested or necrotic in the more chronic lesions. Also, they live for <1 month in cutaneous tissues, and larval death might cause even more necrosis and calcification than a living parasite (20).

Molecular diagnosis can be considered the gold standard. A semi-nested PCR was developed for detection and identification of *Habronema* (*H. microstoma* and *H. muscae*) DNA irrespective of their life cycle stages, with significant repercussions for clinicians. The PCR assay achieved a diagnostic specificity of 100% and a sensitivity of 97% (29, 36, 67, 68). This PCR was developed for the detection of habronematids in gastric form, but it was also able to detect *Habronema* DNA in skin samples from animals with summer sores (69). This can be considered a practical and beneficial approach for veterinarians for the diagnosis of both gastric and cutaneous habronemosis, which are sometimes hard to differentiate from other gastric and skin diseases of equids with comparable clinical signs (29).

PROPHYLAXIS AND TREATMENT

As extensive husbandry conditions seem to be the most effective against habronemosis (70), prophylaxis to decrease the incidence and prevent the reappearance of habronemosis (32, 54, 65) are regular cleaning of the stables and paddocks with proper removal and disposal of manure as part of an integrated fly control plan. Against flies, horses can also be treated with licensed repellents (71) or protected mechanically (using fly nets and blankets); however, some animals do not tolerate fly masks.

Studies focusing on the efficacy of macrocyclic lactones against intestinal strongyles have shown the high efficacy (up to 100 per cent) of ivermectin (200 μg/kg) (72–74) or moxidectin (400 μg/kg) (72–75) against worms in the stomach (49, 65). A more recent study from Brazil demonstrated an efficacy of 92–95%, 98–100%, and 100% of ivermectin, abamectin, and moxidectin, respectively, against *H. muscae* (76). Despite being mostly side data, the efficacy of macrocyclic lactones against gastric *Habronema* was evident, as also confirmed by a focused study that ultimately proved the efficacy of moxidectin against *H. muscae* (77).

For *Habronema* lesions, a single dose of ivermectin did not provide solid evidence of efficacy (49). In some cases, the use of anthelminthic drugs is debatable; for instance, as in the periocular localization the lesions are thought to be a result of local hypersensitivity to dead or dying larvae, the administration of ivermectin may worsen the signs of pruritus (78).

Other drugs (ivermectin, echothiophate, and trichlorfon) have been described for treating cutaneous or mucocutaneous forms (33); in addition, corticosteroids were used to reduce inflammatory hypersensitivity reactions. These molecules can be used mostly in ocular habronematidosis and administered systemically, topically, intra-lesionally or sub-conjunctivally (33, 55).

Surgical debulking intervention is indicated when the medical treatment of summer sores is refractory.

CONCLUSIONS AND FUTURE PERSPECTIVES

Only old-fashioned studies on gastric habronemosis are available in the literature and this would suggest a disappearance or significant reduction of this disease; however, the recent case-reports on cutaneous habronemosis demonstrate that it is maybe not the case.

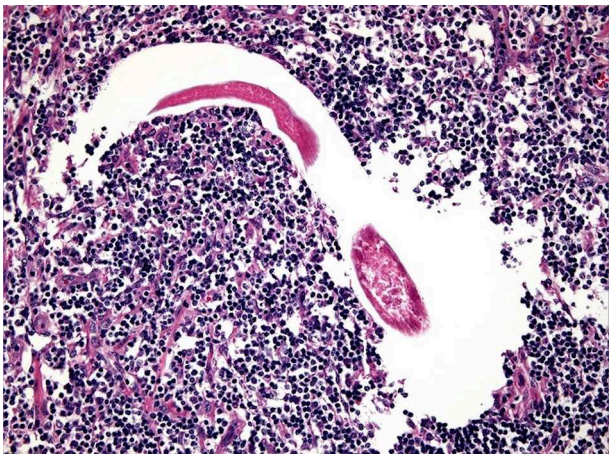

FIGURE 3 | Numerous infiltrates of eosinophils and parasitic forms related to habronematids at histological exam (original, A. Petrella).

Even with swinging academic or technical interests, in its whole the understanding of habronemosis has improved. However, several questions remain unanswered.

A productive multidisciplinary increasingly current research approach that manages the key zones of science, epizoology finding, and treatment, is expected to upgrade the information base and improve the counteraction and control of habronematidosis. Here some tips:

- *Prevalence*: Since clinical cases of cutaneous habronemosis are reported, data on prevalence of gastric habronemosis to which cutaneous habronemosis is related, need to be updated. The lack of information about *Habronema* prevalence is connected with diagnostic tools. Considering that common quali-quantitative copromicroscopic diagnostic approaches are not responsive enough for the detection of *Habronema* eggs and studies at necropsy are time-consuming and difficult to perform, molecular tools must be considered the "gold standard" for future works.
- It is remarkable that PCRs have been designed for the detection of *H. microstoma* and *H. muscae*, but not for *D. megastoma*. Once set up, it could be found out that this latter species is more distributed than expected.
- *Habronema microstoma* vs. *Habronema majus identity*: data on interspecific difference (using *ITS* and *cox1* genes) between *H. microstoma* and *H. majus* have been recently provided (4) but further analysis on the microscopic and genetic make-up of variations among individuals from various geographical areas are needed to ultimately confirm that *H. majus* is a separate species.
- *Host-vector relationship*: The role of *M. domestica* as vector of *H. microstoma* (40, 42) needs to be explored and in particular issues on the cellular, molecular, and/or immunological response of insects related to the possible species-specific susceptibility should be faced. Moreover, due to the different feeding behavior, the role of *S. calcitrans* in trasmitting *H. microstoma* should be further investigated.
- *Anthelminthic resistance*: currently, while there is evidence of increased resistance of the equine cyathostomins and *Parascaris equorum* to various anthelminthics (79), there are no studies on the resistance of *Habronema* species to anthelmintics because these parasites are not included in the standard fecal egg count reduction tests generally used in surveys documenting drug resistance, because their eggs are very difficult to detect with copromicroscopical concentration techniques. Improvements on diagnostic strategies (as above reported) may help to fill also this gap of knowledge.
- *Targeted and alternative treatment*: The widespread anthelmintic resistance calls for alternative control strategies, i.e., to develop novel non-chemicals. The recent *in vitro* experiment on anthelmintic properties of *Verbesina alternifolia* (crown beard) against *H. muscae* which demonstrated by SEM an irreversible degenerative change of the treated worm (80) may be a stimulus to work on this aspect in field condition.

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AB and AG executed a first draft. DT and RP helped draft the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Parasitic Mite Fauna in Asian Poultry Farming Systems

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The ubiquitous presence of hematophagous avian mites threatens the poultry industry in Asia and worldwide, adversely affecting the quality and quantity of eggs and poultry meat produced by affected flocks. This leads to considerable economic loss and welfare-related issues. The role of these blood-feeding arthropods as disease vectors is increasingly recognized as they may carry important zoonotic and epizootic pathogens. The poultry mites, *Dermanyssus gallinae* (Poultry Red Mite—PRM), *Ornithonysus sylviarum* (Northern Fowl Mite—NFM), and *Ornithonyssus bursa* (Tropical Fowl Mite—TFM) are endemic species across the Asian continent. In less frequency, scaly leg mite, depluming mite, and fowl cyst mite were documented. Considering global climate change and the popularity of backyard farming, the incidence of avian mite infestation is expected to increase as Asian production expands. The TFM may start to colonize sub-tropical nations where the seasonal temperature is comparable to tropical regions. Pyrethroids, organophosphates, carbamates, and macrocyclic lactones are licensed acaricides for use in China, Japan, and India. In recent years, the development of acaricide resistance has compromised the efficacy of chemical control measures. Several botanical acaricides based on plant and fungal constituents are being investigated. Judicious integration of multiple approaches to manage poultry mite infestation is advised. In this article, we review the prevalence, geographical distribution, zoonotic potential, and control measures of avian mites in poultry farms in Asia.

Keywords: ***Dermanyssus gallinae*, *Ornithonyssus sylviarum*, *Ornithonyssus bursa*, ectoparasite, zoonosis, one health**

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INTRODUCTION

Mite infestations are of significant concern in the poultry farming industry, affecting the physical and psychological well-being of birds and the quality of egg production. Common avian mites include the poultry red mite (PRM), *Dermanyssus gallinae* (De Geer, 1778; Mesostigmata: Dermanyssidae), the northern fowl mite (NFM), *Ornithonyssus sylviarum* (Canestrini and Fanzago, 1877), and the tropical fowl mite (TFM), *Ornithonyssus bursa* (Mesostigmata: Macronyssidae; Berlese 1888). These mesostigmatic mites are obligatory hematophagous ectoparasites which feed on broilers and egg laying hens (1–3). They frequently colonize the vent feathers, which are warm and humid (3). Hens infested by these poultry mites produced fewer eggs with blood spots, rendering them unsellable (4–7). There are also poultry welfare concerns as heavy infestation could irritate the birds and even death of the flocks due to anemia (1–3). The ability of these mites to survive without attaching to the hosts for a considerable length of time render them difficult to be eliminated.

The rapid human population growth in Asia signifies an ever-increasing demand for food including poultry meat. To date, more than 60% of the world population is inhabited in the Asian continent. The past decades have witnessed a considerable shift of the global poultry market shares from North America and Europe to Asia, where it contributed more than 60% of the global poultry meat and egg production in the twenty-first century (8). Surveys into prevalence and distribution of poultry mites are mostly conducted in North America and Europe and are largely limited to PRM and NFM. To date, there is no review on Asian poultry mites.

In this review, literature search was conducted using title/abstract words including "poultry," "mites," and names of Asian countries without language restriction. Non-English articles were translated into English using Google-translate. The following search engines were used: Google Scholar, Scopus, and Web of Science. We discuss the epidemiology, characteristics and control measures of parasitic mites in Asian poultry farming systems. Other fowl ectoparasites such as lice and ticks are not covered in the current review.

PREVALENCE AND GEOGRAPHIC DISTRIBUTION

Of 50 countries or regions in Asia as defined by the United Nations (9), studies on poultry mites were conducted in 10 countries; including Russia (10–12), China (13, 14), Japan (15–19), Malaysia (20), Myanmar (21), Iran (22–28), India (29, 30), Pakistan (31–34), Vietnam (35), and Saudi Arabia (36). The poultry mite fauna comprises four orders: mesostigmata, sarcoptiformes, acariformes, and trombidiformes.

Dermanyssus galliane

PRM is the most prevalent in Asia and worldwide (2, 3, 37). More than 46% of the farming systems in China, Japan, and Russia were infested by PRM (11, 12, 15). The PRM has variable morphology and genetic plasticity (38). These mites feed on hens occasionally at night and hide in crevices during daytime to avoid acaricide treatment, facilitating its persistence between flocks. Between 2008 and 2009, poultry farmers across 11 provinces in China were invited to participate in a large-scale cross-sectional study on prevalence of ectoparasites in commercial layer farms and broiler breeder farms. Fowl feathers and dust from cracks or crevices in poultry housing facilities were collected as specimens in Ziplock bags (12). More than 800 specimens were collected from farms collectively housing 5.5 million layer hens and 4.2 million of parent hens, representing more than 50% of the poultry production in China. Of 833 specimens, more than 80% were positive for at least one mite species or other ectoparasites. The PRM was present in 64% of commercial laying hens and 37% of breeder hens. Likewise, our recent review indicated that the prevalence of PRM was higher in layer hens (85.2%) than in broilers (0.6%) (1). In Iran, PRM was also highly prevalent in the majority of farms (22–24). Of note, inspection of eight caged layer farms and four breeder flock premises yielded PRM in all of the facilities (22). It should be noted that the latter study sampled from farms where farmers noticed ectoparasite infestation in their farms. This may explain the high prevalence in that study (22).

The prevalence rate of PRM in Asia was similar to that in Europe (1) and Africa (39–42). Although some studies reported a low prevalence of PRM and other mite species, it should be noted that these studies lacked random sampling and were largely limited to a few farms, which may not be representative of the countrywide situation. The dry and hot tropical climate in some Asian countries may render arthropods susceptible to dehydration and thus hindering their persistence in the environment. Temperature of the barns may not be well-controlled due to minimal resources available in some developing countries. For instance, the prevalence of PRM in North Africa and Iran, the prevalence of PRM varied across studies between 11% and up to 100% (23–25, 41, 42).

Ornithonyssus spp.

The NFM and TFM are the important *Ornithonyssus* spp. responsible for poultry infestation. They are known to be prevalent in temperate and tropical regions, respectively. In Myanmar, four out of five premises were inhabited by either NFM or TFM (21). Interestingly, no PRM was isolated from any of the farms (21). In China, NFM was more frequently identified in breeders (46.9%) than in commercial layer hens (22.7%) (13). Another Chinese study was conducted in seven poultry housing premises across Hainan Island (China), where the climate ranged from tropical to subtropical (14). This study reported a prevalence rate of NFM as 42.8%, which was similar to another study conducted in Northern Chinese poultry farms (46.9%) (13). In the tropical Hyderabad region of India, TFM was frequently isolated from cage fittings, beneath feed troughs, fastening clips, under egg conveyer belts, and under manure belts across five breeding and caged layer poultry facilities (30). In Iran, TFM was found in more than 8% of the breeder flocks in the northern region warm and humid weather (23, 25). Likewise, in hot climate location in South America, such as Brazil, the TFM was the most commonly found mite species ($n = 24{,}274$) (43).

Knemidocoptes and *Laminosioptes*

Sarcoptiforms comprise scaly leg mite (SLM), *Knemidocoptes mutans,* and depluming mite, *Knemidocoptes gallinae*. These are related mite species that burrow under the skin and lay eggs in the subcutaneous layer. Depluming mites mostly colonize ventral wings and the abdomen. The affected feathers became more susceptible to breakage. In addition, depluming mites burrow under the skin causing irritation to the birds. Most of the birds would try to pull out the affected feathers and thus creating lesions. In Russia, between 17.7 and 76% of the domestic fowls were infested by either SLM or depluming mite (12). In India, skin scraping of a male Aseel chicken with whitish film layer on legs and focal sloughing of the epidermis revealed the presence of larval and nymphal stages of the scaly leg mite.

The acarine *Laminosioptes cysticola* (Acariformes: Laminosioptidae, Vizioli, 1870), the fowl cyst mite (FCM), was reported in broiler farms in India (26). The research team monitored a group of 400 broilers over 3 years estimated 2.75%

TABLE 1 | Summary of studies with prevalence estimates on parasitic mite fauna in Asian poultry farming systems.

Country (References)	Sample[a] mean flock size or range	Mite	Prevalence (%) by production system		
			Cage	Backyard	Unknown
Russia (12)	600	*D. gallinae*	-	55.7	-
		K. mutans	-	17.7	-
		K. gallinae	-	17.7	-
China (13)	6,360,200 layers	*D. gallinae*	64.1	-	-
		O. sylviarum	22.7	-	-
	5,534,300 breeders	*D. gallinae*	36.8	-	-
		O. sylviarum	46.9		-
China (14)	281,000	*O. sylviarum*	42.8	-	-
Japan (16)	700	*D. gallinae*	-	-	46.9
		O. sylviarum	-	-	17.3
Myanmar (21)	20 farms	*O. bursa*	-	-	10
		O. sylviarum	-	-	15
Iran (24)	600	*D. gallinae*	-	-	11
Iran (25)	600	*K. mutans*	-	7	-
		D. gallinae	-	26.3	-
		O. bursa	-	8.5	-
Iran (26)	400 broilers	*L. cysticola*	-	-	2.75
Pakistan (33)	Not reported	*D. gallinae*	14	-	-
		Laelaptidae	16	-	-
		Macrochelidae	57	-	-

[a]*Sample is presented in mean flock size or range unless otherwise stated by the authors. The types of flocks are stated according to the description in references.*

prevalence of FCM by microscopic examination. FCM formed cysts underneath the poultry skin as the female mites laid eggs. Superimposed bacterial infection frequently ensued (26). While the pathogenicity of FCM does not always lead to mortality, this mite species considerably distort the quality of meat in broilers rendering it unsellable to markets.

The epidemiology of poultry mites across Asia are summarized in **Table 1**. Prevalence and distribution is depicted in **Figure 1**.

CO-OCCURRENCE OF MITE INFESTATION WITH OTHER ECTOPARASITES

Mite infestation in poultry frequently co-occur with other ectoparasites. It was reported that as much as 70% of commercial hens were parasitized at the same time by mites and other ectoparasites such as lice, fleas, and soft ticks (24, 25, 27). In Ethiopia, more than 34% of SLM were present in flocks from which fleas and lice were also isolated (40). Likewise, PRM was isolated from poultry farms together with ticks and lice (32). In contrast, in a Scottish smallholding where an adult cockerel and two young chicks were dead, the birds were found to be infested with PRM and depluming mite without other ectoparasites (44). Accordingly, we hypothesized that the ecological competition between mites and other ectoparasites such as lice, fleas and ticks may be more important in Asia than in Europe.

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION

Numerous studies employed microscopic morphology for the sole species identification method. Considering morphological similarity between different mite species and that morphological characteristics may be present shortly in a particular developmental stage, microscopic analysis alone may be limited. Differentiation between NFM and PRM requires specialized keys for identification of distinct characteristics in the dorsal shield, epigynal shields, and the chelicera (45). The advance of molecular technology in the last decade such as phylogenetic analysis provided evidence of epidemiological relatedness between mites. This relationship was not possibly determined by microscopic observation. The most commonly used polymorphic gene for this purpose is mitochondrial *cytochrome oxidase subunit 1* (COI) (14, 15, 46).

Applying COI gene sequencing could improve the accuracy of mite species prevalence estimation and reveal the possible transmission of mite species. For instance, in Japan, sequencing COI gene of 239 specimens led to identification of 28 haplotypes of PRM (15). Of these, two haplogroups were found identical to the European PRM clones. This information suggested possible importation of these mite haplogroups from Europe to Japan through international trade of infested chickens and poultry farm materials (15). In China, Bhowmick et al. (14) sequenced the COI marker gene to determine the prevalence of NFM. They

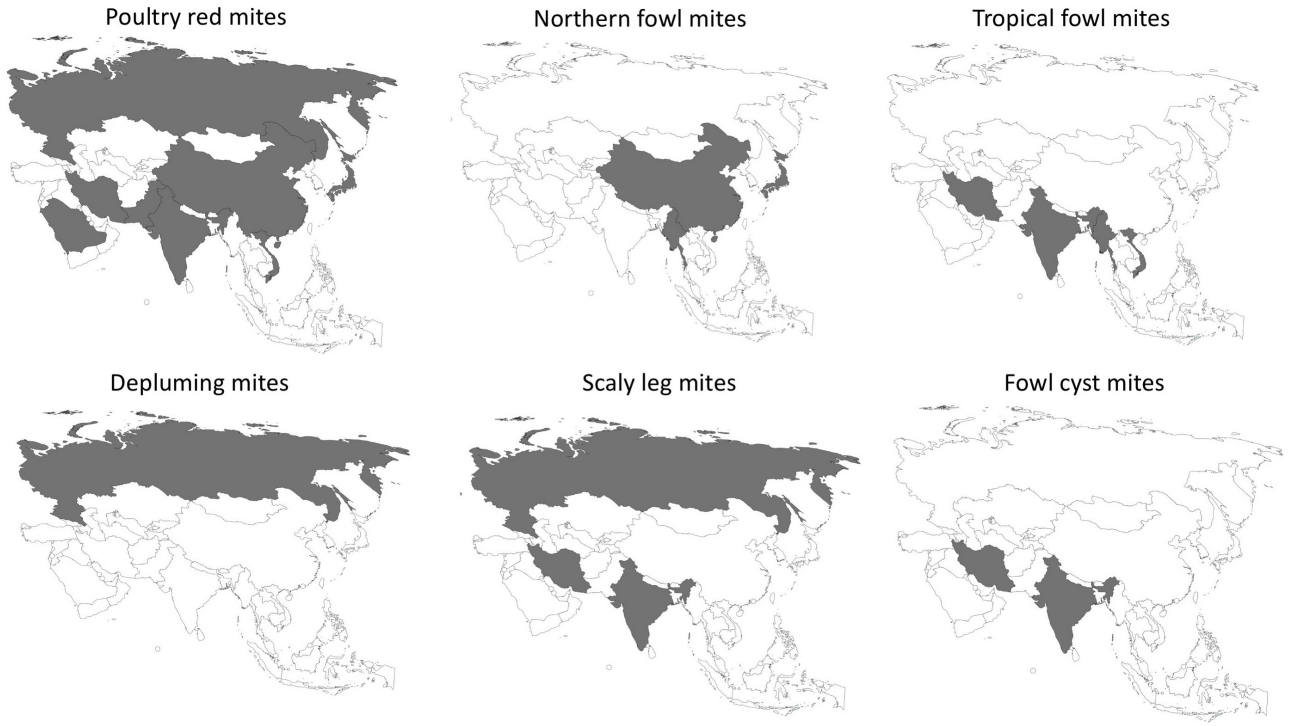

FIGURE 1 | Geographical distribution of mite fauna in Asian poultry farming system (12–36).

found that all NFM isolates in Hainan were closely related. The majority of the haplotypes differed from each other by only single nucleotide (14).

POTENTIAL PHORETIC ASSOCIATION BETWEEN POULTRY MITES, WILD BIRDS, AND FLIES

In many developing countries, the biosecurity level of poultry farms is low without proper hygiene. In Iran, for instance, more than 20% of the native fowls carried the poultry depluming mites (34). The commercial flocks were kept in free-range in a small-scale facility where they are at risk of contact with infested wild birds (34). Wild birds appeared to play a role in the dissemination of PRM among domestic birds in Brazil but not in Europe and Australia (47). It should be noted that the transmission of poultry mites between wild birds and commercial flocks remains elusive for other poultry mites. Further molecular characterization of mite fauna in commercial hens and proximal wild fowls in Asia will be required to confirm the hypothesis.

Diptera (true flies) are commonly found in poultry farms. Of 13,343 *Musca domestica* collected from poultry premises, 10 families of mites at different developmental stages were found. The most common one was *Macrocheles muscaedomesticae* (Mesostigmata: Macrochelidae), followed by Trombidiformes (Trombidiidae, Pygmephoridae, Tydeidae, Tarsonemidae, and Erythraeidae) and Sarcoptiformes (Pyroglyphidae, Histiostomatidae) (20). This study led to the hypothesis of insects as potential phoretic vectors for transmission of poultry mites.

ONE HEALTH-RELATED ISSUES

Beyond the poultry farming industry, the avian mites are of both veterinary and medical concerns due to their potential roles as vectors for bacterial and viral pathogens (48, 49) and possible host range expansion (50). Although the absolute vector competence of poultry mites has not yet been confirmed, the presence of epizootic and zoonotic pathogens in these mites is evident. The PRM is capable of carrying avipox virus, fowl adenovirus, Marek's disease virus, *Erysipelothrix rhusiopathiae*, *Salmonella enterica*, *Mycoplasma synoviae*, and *Mycoplasma gallisepticum* (17, 51). The proximity between poultry, domesticated birds, companion animals, and humans may facilitate alternate host adaptation of mesotigmatic mites (50). There is also controversy on whether different *Dermanyssus* subspecies infest chicken and pigeons (Personal communication). PRM has also been reported in cats and dogs (52, 53) and in humans as clinical dermatitis (also known as gamasoidosis) (54–56). Human cases of PRM infestations in Europe have been extensively reviewed (57). Other mesotigmatic mites including NFM and TFM have been isolated from people whose apartments were proximal to abandoned bird nests (54). In Asia, there were reports of human cases of NFM dermatitis in Japan (58) and PRM pruritus in Iran (55)

INFESTATION CONTROL MEASURES

Chemical Methods

Traditionally, poultry mite management in livestock production relies on chemical acaricides. Chemical acaricides include organophosphates (e.g., Dichlorvos), pyrethroids (e.g., Cypermethrin, Deltamethrin), carbamates (Carbaryl), and macrocyclic lactones (Abamectin, Milbemectin). All of these were reported being used in mainland China (13) and Indian poultry farms (59). These groups of acaricides are also licensed for use in the veterinary market in Hong Kong (60) and Japan (18).

In mainland China, the majority of poultry farms (>50%) used either pyrethroids or organophosphates alone. More than 25% of poultry farmers do not re-treat their birds with acaricides within 2 weeks after the first treatment. This practice may lead to recolonization by the residual larva and promote chemical tolerance in mites. Although avermectins (i.e., abamectin and ivermectin) were not authorized for use in poultry flocks in mainland China, 63% farmers routinely supplement the bird feed with 1–2 ppm abamectin or ivermectin. This highlights the importance of education of farmers on appropriate use of acaricides and adherence to national guidelines.

Acaricide resistance in mites develop either as a result of increased metabolic breakdown of the acaricide or through acquisition of genetic mutation encoding an altered target with reduced affinity to the acaricide (61, 62). In Japan, 19.5% PRM in poultry farms showed resistance to three classes of acaricides, namely carbamates, pyrethroid, and organophoshates (18). Remarkably, the prevalence of PRM resistance to all commercially available acaricides increased from 13.7% in 2007–2010 to 18.3% in 2011–2013 (18). The occurrence of resistance against carbamate and combination insecticide [fenitrothion, permethrin, phthalthrin] appeared to be less frequent than those of other acaricides and insecticides (18). The underlying mechanism contributing to this phenomenon remains elusive.

To combat acaricide resistance in poultry mites, liquid preparation of diatomaceous earth, Silicon dioxide, was investigated. The insert dust is able to physically immobilize PRM and inhibits its locomotion. Coupling with mechanical cleaning, field trials confirmed that spraying 82% silica-containing Fisiocontrol (VetScience Bio Solutions) reduced up to 94% of the PRM population by 42 days after the first application (63).

Biological Methods

Several plant extracts have been tested for use as botanical acaricides against poultry mites. In field trials, 10% garlic extract could considerably reduce mite infestation rate and restore erythrocyte and leucocyte counts in flocks (64, 65). Garlic extract is effective against NFM and PRM (28, 64). Some garlic-based products such as Garlic Barrier (Garlic Research Labs, United States) and Breck-a-Sol (ECOspray, United Kingdom) have already commercialized for controlling NFM and PRM (2). Laboratory *in vitro* studies on plant constituents revealed promising results of using plant-derived essential oils against PRM and NFM (66–69). Extracts from Samandua, Lychee, and Clove showed promising contact toxicity and vapor toxicity against PRM (67). Thyme and cade oils are effective against NFM (68).

Another biological control method is the use of entomopathogenic fungi such as *Aspergillus oryzae* and *Metarhizium* spp. against PRM (70, 71). Inoculation of *Metarhizium* strains at a concentration of 5×10^5 condidia per cm^2 reduced adult mite population by 56–95% in seven days (71). A comparable inoculum size of *A. oryzae* led to 10% higher mortality rate in treated PRM compared to the placebo group (70). Following field trials in poultry farms and appropriate control of dosage delivery, these fungal species may provide alternative options for biopesticides in the future.

Vaccination of poultry flocks against acarine has been increasingly recognized as a possible solution for arthropod control (2). Nonetheless, vaccine development is time-consuming and requires a thorough understanding of local epidemiology of poultry mites (2).

Recombinant akirin (Deg-AKR), calumenin (Deg-CALU), and *Rhipicephalus microplus* Subolesin (Rhm-SUB) have been identified as potential vaccine candidates (72, 73). Safety and technical issues have to be addressed before introducing arthropod vaccine into the poultry industry.

Physical Method

The design of premises has long been recognized as an important determinant of ectoparasite infestation in poultry farms. The open systems such as free-range and backyard housing facilities are more prone to mite infestation as compared to the traditional caged system (1). Experimental studies and field trials suggested the possibility of using light regimen and gas for managing mite infestation. The population growth rate of PRM in rearing system under prolonged darkness (1: 23 h L:D) was three-fold higher than that with conventional lighting regimen (12: 12 h L:D) (74). Application of carbon dioxide could induce asphyxiation and thus reduce mite population by 85% within 24 h and 100% by 120 h (75).

Recently, the concept of integrated pest management has been extrapolated into use in poultry farming systems. The combination of chemical treatment, physical environmental control, and cultural interventions to control mite burden in poultry farming may reduce the risk of developing acaricides resistance and preserve the effectiveness of these armamentarium in the years to come. This kind of integrated mite infestation management program has been reviewed elsewhere (59).

CONCLUDING REMARKS

Given the paucity of well-designed epidemiological studies on poultry arthropods in Asia, the prevalence of mite species circulating in farming systems remains elusive. The popularity of small-scale open farming systems in Asia may complicate the implementation of effective and affordable treatment strategies. Poultry mites frequently co-infest the same flocks with other ectoparasites. Further studies on the epidemiology of poultry mites and the interaction between mites and other ectoparasites are warranted to justify the use of appropriate control measures.

AUTHOR CONTRIBUTIONS

OS conceived the review topic, provided intellectual input, and critically revised the manuscript for submission. OS and JH performed literature searches. JH prepared the first draft of the manuscript and processed the data for the published figures. All authors contributed to the article and approved the submitted version.

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Characterization of *Neospora Caninum* Microneme Protein 26 and Its Potential Use as a Diagnostic Marker for Neosporosis in Cattle

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The apicomplexan parasite *Neospora caninum* causes neosporosis, an illness that leads to abortion or stillbirth in cattle, causing massive economic losses to the livestock industry. Rapid and viable diagnosis is the premise of prevention and control for neosporosis. In this study, we screened a new microneme protein 26 (NcMIC26) through western blot and mass spectrometry identification from the excretory secretion antigen (ESA) of *N. caninum* tachyzoites. NcMIC26 is subcellularly localized to the microneme of parasites. NcMIC26 is a specific antigen of *N. caninum* and has no cross-reaction with *Toxoplasma gondii*. Therefore, NcMIC26 has the potential to be a candidate diagnostic antigen for neosporosis. To test this hypothesis, recombinant NcMIC26 (rNcMIC26) was expressed in *Escherichia coli* (*E. coli*), and an indirect ELISA for detecting anti-*N. caninum* antibodies in cattle was established. Compared with that of the indirect immunofluorescent antibody test (IFAT), the positive coincidence rate of the ELISA based on rNcMIC26 was 76.53% (75/98), which was higher than that of an ELISA based on rSRS2 (66.33%), and the negative coincidence rate was 84.62% (33/39). It is noteworthy that 30 positive samples confirmed by IFAT were consistent with the rNcMIC26 ELISA but were negative by the rNcSRS2 ELISA. Our research illustrated that NcMIC26 is a dependable diagnostic marker for the serodiagnosis of *N. caninum* infection in cattle and could be utilized as a supplementary antigen for missed detection by NcSRS2.

Keywords: ***Neospora caninum*, ESA, microneme protein 26, ELISA, cattle**

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INTRODUCTION

Neospora caninum is an obligate intracellular apicomplexan parasite that is the etiologic agent of neosporosis for a variety of animals, for which canids are the definitive hosts (1). The disease tends to be globally distributed and is most serious in cattle (2). Abortion is the main clinical symptom of infection, and neosporosis is one of the main causes of cattle abortion worldwide (3). Reproductive loss is the main clinical outcome of neosporosis in cattle and a major reason for the economic impact on the dairy and beef cattle trade (2). In the absence of an effective treatment or vaccine against bovine neosporosis, control of the disease depends on an accurate diagnosis of neosporosis-infected cattle for timely treatment or early elimination of livestock and other farm management measures.

The recombinant antigens used or validated for indirect ELISA are based on different biological function-associated antigens, including the surface antigens NcSAG1 (4), NcSRS2 (5), NcP40 (6), NcSAG4 (7), and rNcSRS9 (8); dense granule antigens NcGRA2 (9), NcGRA6 (10), and NcGRA7 (11); microneme antigen NcMIC10 (12); and other antigens, such as *Neospora* profilin (13). The NcSRS2 antigen is the most widely used and shows excellent diagnostic parameters (14). However, it is hard to examine the antibody response simultaneously against different antigens to which a host is differentially exposed depending on the stage (the acute or chronic stage) of *N. caninum* infection by using single antigens in ELISA. Similar to most other apicomplexan parasites, the process of invasion is necessary for *N. caninum* to survive and replicate within the host (15), and the proteins discharged by tachyzoites, known as the excretory secretion antigens (ESAs), are the most common targets of host immune reactions; recognizable proof of ESAs included in invasion may be valuable for revealing the critical target for and the prevailing antigen of *N. caninum* (16). In the present study, we screened a microneme protein 26 (NcMIC26) from *N. caninum* ESAs. The localization of NcMIC26 is in the microneme of parasites, and it partially colocalizes with NcMIC4. Recombinant NcMIC26 was expressed in *Escherichia coli*; a reliable, sensitive, and specific diagnostic test based on recombinant NcMIC26 was developed; and its diagnostic potential in an ELISA was evaluated.

MATERIALS AND METHODS

Parasites and Cell Cultures

N. caninum Nc-1 strain tachyzoites were propagated in African green monkey kidney (Vero) cells cultured in Dulbecco's modified Eagle's medium (DMEM) (M&C, China) containing 25 mM glucose and 4 mM glutamine and supplemented with 2% fetal bovine serum (FBS, Gibco, USA). Cells were incubated at 37°C with 5% CO_2 in a humidified incubator.

Preparation of *N. caninum* Tachyzoite ESA and Soluble *N. caninum* Lysate Antigen

To find a new *N. caninum* diagnostic antigen, we employed mass spectrometry-based proteomics to identify proteins present in the *N. caninum* tachyzoite using two different approaches. The first approach was identifying the proteins present in the tachyzoite-secreted fraction (ESA). ESA were obtained according to a method involving *Toxoplasma gondii* that was previously described (17). Briefly, the tachyzoites were harvested from Vero cell cultures. Twenty-seven-gauge needles were used to disrupt the cells, and lysates were filtered through a 5-μm syringe filter. The purified tachyzoites were washed three times in DMEM by centrifugation at 900 × g for 10 min. The freshly purified tachyzoites were incubated (5×10^7 parasites/mL) in a serum-free medium (DMEM) at 37°C for 3 h and cooled for 10 min on ice. The supernatant separated from the parasites and containing ESA was collected by centrifugation at 20,000 × g for 10 min at 4°C. The parasites were lysed using a RIPA buffer (Beyotime, China) supplemented with a cocktail of protease inhibitors (Sigma, USA).

The second approach was to identify the secreted proteins in the culture medium of intracellular tachyzoite cultures. Nc1 tachyzoites were inoculated in Vero cells. The medium was discarded after 3 days before its egress and washed with PBS three times, and then a serum-free DMEM was cultured at 37°C for 24 h. A dialysis bag (Harveybio, China) was used to concentrate the collected secreted proteins from intracellular culture. The medium collected from the Vero cell culture served as the control.

Identification of *N. caninum*-Specific Antigen From Tachyzoite ESA

The polyclonal antiserum against *N. caninum* or *T. gondii* was generated in mice or rabbits using tachyzoites lysate antigen as described previously (18). Six- to eight-week-old female BALB/c mice and 2-month-old rabbits were purchased from the Academy of Military Medical Sciences Laboratory Animal Center (Beijing, China). Mice and rabbits were immunized subcutaneously every 2 weeks with 100 μg (mice) or 2 mg/kg (rabbits) tachyzoites lysate antigen in an equal volume of Freund's complete adjuvant (Sigma, USA) for the first injection and 50 μg (mice) or 1 mg/kg (rabbits) for the second and third injections. The anti-*N. caninum* or anti-*T. gondii* sera were collected 10 days after the final immunization. Animal experiments were conducted according to the institutional guidelines for animal ethics.

SDS-PAGE and western blot were used to identify the collected *N. caninum* proteins, which were performed as previously described (6). The *N. caninum* tachyzoite ESA and lysate samples were loaded into a 12% SDS-PAGE gel with equal loads. Separated protein bands were visualized in gels by silver staining according to the manufacturer's protocol for the Silver Stain Kit (Beyotime Biotechnology Co., Ltd., China) or transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA) together with a visible prestained protein marker (TransGen Biotech Co., Ltd., China) after electrophoresis (Bio-Rad). The membranes were blocked with 5% (w/v) skim milk (BD Difco, USA) in PBS for 1 h at 37°C, rinsed with a washing buffer, and incubated with a mouse polyclonal antiserum against *N. caninum* (1:400) or a rabbit polyclonal antiserum against *T. gondii* (1:400) for 1 h at 37°C. The blots were washed five times with PBST (1% Tween-20), followed by incubation with horseradish peroxidase (HRP)-labeled goat antimouse IgG (H + L) (1:5,000, Sigma, USA) or HRP-labeled goat antirabbit IgG (1:10,000, Sigma, USA). Finally, enhanced chemiluminescence reagents (CoWin Biotech Co., Ltd., China) were used to observe the reaction bands after 5 s of exposure time.

Abbreviations: *N. caninum*, *Neospora caninum*; *T. gondii*, *Toxoplasma gondii*; *E. coli*, *Escherichia coli*; ESA, excretory secretion antigen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LC-MS/MS, liquid chromatography mass spectrometer; WB, western blotting; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; IFAT, indirect immunofluorescent antibody test; IPTG, isopropyl-β-d-thiogalactoside; MIC, microneme protein; SRS, surface antigen related protein; SAG, surface antigen; GRA, dense granule protein; DHFR, dihydrofolatereductase; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate.

According to the western blot results, the corresponding specific bands (realized by anti-*N. caninum* but not anti-*T. gondii* antibodies) in SDS-PAGE protein strips were cut out for mass spectrometry (MS) and protein identification (Beijing Protein Innovation Co., Ltd., China) to obtain the corresponding peptide information and determine the *N. caninum*-specific antigen. Analysis of the DNA and protein sequences was performed using The Toxoplasma Genomics Resource (ToxoDB) website (https://toxodb.org/toxo/) and the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/). Conserved domains are available on the NCBI Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Gene Cloning, Recombinant Protein Expression, and Purification

Total RNA was extracted from purified *N. caninum* tachyzoites, reverse-transcribed (TransGen Biotech Co., Ltd., China), and used as a template for PCR. Primers were designed for the region 271–1,014 bp of the NcMIC26 RNA sequence (ToxoDB, NCLIV_033690), with low similarity with the homologous gene in *Toxoplasma gondii*. The primer sequences were as follows—forward primer: 5′-AGCAAATGGGTCGC@@UGGATCCLINE @@GTTCTGGATTTCATAGACTTGG-3′, containing a *Bam*HI site, and reverse primer: 5′-TCGAGTGCGGCCGC@@UAAGC TTLINE@@CGAAGTCCATTCGCCCCACGTT-3′, containing a *Hind*III site. The truncated NcMIC26 gene was amplified with 2× ExTaq Mix polymerase (TransGen Biotech Co., Ltd., China), and PCR was performed using the following procedure: 95°C for 5 min; followed by 35 cycles at 95°C for 30 s, 57°C for 1 min, and 72°C for 1 min; and extension at 72°C for 10 min.

The resultant PCR products were ligated into the pET-28a expression vector backbone after *Bam*HI and *Hind*III double enzymatic digestion. The insert sequences were sequenced and aligned to the NcMIC26 gene sequence reported previously (19). The recombinant plasmid was named pET-28a-NcMIC26.

The recombinant plasmid was transformed into *Escherichia coli* Transetta (DE3). Expressed as a His tag fusion protein following induction with 0.8 mM IPTG for 6 h at 37°C, rNcMIC26 was purified by column chromatography using Ni-NTA Superflow columns and stored at −80°C until use.

NcMIC26 Subcellular Localization

NcMIC26 endogenous epitope tags were generated as described previously (20). Briefly, the plasmid pNc_Cas9CRISPR::sgNcMIC26 and the plasmid pLIC-HA-DHFR-NcMIC26 (a template of homologous repair amplicon) were constructed, and the primers are listed in **Table S1**. Then, the parental Nc1 strain was cotransfected with the plasmid pNc_Cas9CRISPR::sgNcMIC26, and a linearized homologous repair was completed. The transgenic parasites were grown under pyrimethamine (1 μM) selection pressure to the third generation and then screened to confirm the purity of the selected strains. The selected strain was named NcMIC26-HA.

IFAT was used for NcMIC26 subcellular localization in parasites as previously described (20). Briefly, 1×10^5 *N. caninum* tachyzoites were seeded onto HFFs that were already arranged on glass coverslips in 12-well-plates (Corning costar, USA). Infected cells were incubated at 37°C with 5% CO_2 for 30 h, fixed for 30 min in 4% formaldehyde, permeabilized with 0.25% Triton X-100 for 15 min, and then blocked with 3% bovine serum albumin (BSA) for 30 min. Subsequently, the cells were incubated with a mouse anti-HA monoclonal antibody (1:50, Sigma-Aldrich) and a rabbit antiNcSRS2 polyclonal antibody [1:500, (20)], the primary antibodies were detected with FITC-conjugated goat-anti mouse IgG (H + L) (1:100, Sigma, USA) and Cy3-conjugated goat-anti rabbit IgG (H + L) (1:100, Sigma, USA), respectively, and the nuclear DNA was stained with DAPI (1:200, Sigma, USA). Finally, a Leica confocal microscope system (Leica, TCS SP52, Germany) was used to obtain images.

Specificity Analysis

The expression and purity of recombinant protein were analyzed by SDS-PAGE electrophoresis and visualized by Coomassie blue staining. Besides, SDS-PAGE and western blots were used to confirm the reactogenicity and antigenic specificity of *N. caninum*. Western blots were followed as described above; rNcMIC26 was subjected to electrophoresis and transferred electrophoretically onto a PVDF membrane. After blocking, the blots were incubated with mouse polyclonal antiserum against *N. caninum* (1:400), mouse *N. caninum*-negative serum (1:400), mouse polyclonal antiserum against *T. gondii* (1:400), and mouse anti-His monoclonal antibody (1:500) for 1 h at 37°C. After washing, the cells were incubated with a horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (H + L) secondary antibody (1:5,000).

Indirect ELISA

Indirect ELISA tests based on the purified recombinant protein NcMIC26 were developed as previously described (21). Optimal dilutions of the antigen and bovine sera were determined by checkerboard titration. *N. caninum*-positive and negative sera samples were employed to each assay. We diluted the His-fused rNcMIC26 in a coating buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6) to a final concentration of 1 μg/well, added it to 96-well flat-bottom plates (Guangzhou Jet Bio-filtration Co., Ltd., China), and incubated it at 37°C for 1 h and then at 4°C overnight. After four washes with a washing buffer (PBS containing 0.1% Tween 20), the plates were blocked with a blocking buffer (PBS containing 5% horse serum) at 37°C for 1 h. The plates were washed four times, the cattle sera were diluted in a diluent solution (PBS containing 2% horse serum, 1:200), and 100 μl was added to each of the duplicate wells of the ELISA plate and incubated for 1 h at 37°C. The plates were rinsed as before and incubated with the HRP-conjugated goat anti-bovine IgG antibody (Southern Biotechnology Associates, Inc., USA) diluted in a diluent solution (1:25,000) at 100 μl/well for 0.5 h at 37°C. Finally, the plate was rinsed, and bound antibodies were detected by incubation with a 100 μl/well tetramethylbenzidine (TMB) substrate (M&C Gene Technology Co., Ltd., China) and color rendering at room temperature for 10 min. The reaction was stopped with a stop solution (2 M sulfuric acid, 50 μl/well), and the absorbance was measured at 450/630 nm in an ELISA plate reader (Bio-Rad). Every experiment was repeated three

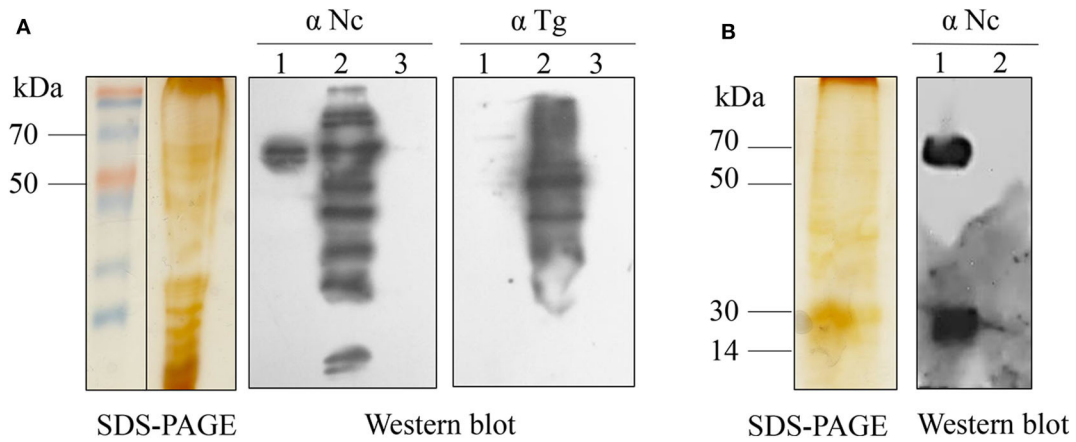

FIGURE 1 | SDS-PAGE and western blot analyses of *N. caninum* ESAs. **(A)** SDS-PAGE and western blot analyses of *N. caninum* tachyzoite-secreted fraction ESAs. Lane 1, *N. caninum* tachyzoite-secreted fraction ESA; lane 2, *N. caninum* tachyzoite lysate; lane 3, DMEM control. Western blot using anti-*N. caninum* serum (1:400) or anti-*T. gondii* serum (1:400). **(B)** SDS-PAGE and western blot analyses of *N. caninum* tachyzoite-secreted protein for intracellular culture. Lane 1, *N. caninum* tachyzoite secreted protein from intracellular culture; lane 2, Vero cell control. Western blot using anti-*N. caninum* serum (1:400).

times. The cutoff point was determined as the mean $OD_{450/630nm}$ for *N. caninum*-negative sera kept in our laboratory (n = 30) plus three standard deviations. The positive antibody of *N. caninum* was found in samples whose OD value ≥ cutoff point, and the sample OD value < cutoff point was negative for *N. caninum*.

Data Analysis

The sera used in this study were as follows: gold-standard panel sera consisting of IFAT-defined negative sera (n = 39) and IFAT-defined positive sera (n = 98) from cattle. The number of positive or negative sera samples of the two diagnostic methods was counted manually. Statistical analysis of the data was performed using SPSS 22. Kappa coefficient was used to evaluate the level of agreement between ELISA methods (based on NcSRS2 or NcMIC26) and the gold standard (IFAT). The specificity and sensitivity of detecting *N. caninum* serum antibodies by ELISA were determined using the following formulas: sensitivity = (number of ELISA test-positive sera/number of IFAT test-positive sera) × 100%; specificity = number of ELISA test-negative sera/number of IFAT test-negative sera) × 100%). Indirect ELISA methods based on the rNcMIC26 and rNcSRS2 (5) antigens were used to detect these sera, and the relevant data were compared and analyzed.

Ethics Statement

The experiments with animals in this study were performed strictly according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China and approved by the Institutional Animal Care and Use Committee of China Agricultural University (under the certificate of Beijing Laboratory Animal employee ID: CAU20161210-2).

RESULTS

Identified NcMIC26 From ESA of *N. caninum*

The collected ESA proteins were separated by SDS-PAGE, and western blot was used to identify antigen reactivity and specificity (**Figure 1**). Three specific protein bands (50–70 kDa from **Figure 1A**; 14–30, 50–70 kDa from **Figure 1B**) of *N. caninum* showed no reaction with *T. gondii*-positive serum. The tryptic peptides were analyzed by LC-MS/MS, corresponding to a total of 121 proteins after the appropriate cutoff filters were applied to the results (**Table 1**). First, we excluded proteins that were inconsistent with their corresponding band mass. Then, we mainly screened for proteins related to the three major secretory organelles and, eventually, determined NcMIC26 to be a candidate antigen of *N. caninum*, which was present in the tachyzoite-secreted fraction ESA (50–70 kDa from **Figure 1A**).

The gene sequence and the amino acid sequence of *N. caninum* NcMIC26 were obtained from ToxoDB (http://toxodb.org/toxo/; Gene ID: NCLIV_033690). The full-length NcMIC26 protein is composed of 756 amino acids, and a hydrophobic region at the N-terminus has characteristics of a signal peptide (1–33 amino acid). The mature protein has a predicted molecular weight of 80 kDa. Two putative transmembrane spanning helixes were found between amino acid residues 12–34 and 687–709, near the N-terminus and C-terminus of NcMIC26, respectively. In addition, the protein contains a Von Willebrand factor type A (vWA) domain (67–248) and five thrombospondin type 1 (TSP-1) repeat regions (261–325, 330–388, 394–449, 454–510, and 516–575). The NcMIC26 amino acid sequence aligned to other similar Apicomplexa proteins; the deduced NcMIC26 amino acid sequence was 45% identical to that of *N. canimum* MIC2 (NCLIV_022970), 44% identical to that of *T. gondii* MIC2 (TGGT1_201780), 59% identical to that of *Cystoisospora suis* microneme protein (CSUI_022748), and 52.76% identical to that of *Sarcocystis neurona SO SN1* syntenic protein (SRCN_7088).

TABLE 1 | *N. caninum* antigens from the tachyzoite ESA and tachyzoite-secreted proteins by LC-MS/MS.

Gene ID	Weight	Name	Orthologous organism	Orthologous gene
NCLIV_021050	93,650	Unnamed protein product	*Toxoplasma gondii ME49*	Subtilisin SUB1
NCLIV_001970	67,066	Predicted rhoptry protein kinase (ROPK)	*Toxoplasma gondii ME49*	Rhoptry protein ROP7
		Predicted rhoptry kinase, subfamily ROP24	*Toxoplasma gondii ME49*	Rhoptry protein ROP4
NCLIV_012920	43,208	Predicted rhoptry kinase, subfamily ROP23	*Toxoplasma gondii GT1*	Rhoptry family protein ROP40
NCLIV_060730	61,541	Predicted rhoptry protein kinase (ROPK)	*Hammondia hammondi strain H.H.34*	Rhoptry protein ROP5
		Predicted rhoptry kinase, subfamily ROP32	*Toxoplasma gondii ME49*	Rhoptry protein ROP5
NCLIV_043270	50,154	Putative microneme protein MIC1	*Toxoplasma gondii ME49*	Microneme protein MIC1
NCLIV_011730	59,828	Predicted rhoptry pseudokinase, subfamily ROP26	*Toxoplasma gondii GT1*	Rhoptry kinase family protein ROP26
			Hammondia hammondi strain H.H.34	Rhoptry kinase family protein ROP26
			Sarcocystis neurona SN3	Rhoptry kinase family protein ROP26
NCLIV_031550	48,172	Predicted rhoptry kinase	*Cystoisospora suis strain Wien I*	Rhoptry kinase family protein rop37
NCLIV_028170	63,997	ROP40	*Toxoplasma gondii GT1*	Rhoptry kinase family protein ROP20
NCLIV_045870	22,773	GRA3	*Toxoplasma gondii ME49*	Dense granule protein GRA3
NCLIV_036400	19,849	GRA1	*Toxoplasma gondii ME49*	Dense granule protein GRA1
NCLIV_033690	82,521	MIC26	*Cystoisospora suis strain Wien I*	Microneme protein
			Toxoplasma gondii GT1	Microneme protein MIC2
NCLIV_002940	66,537	Putative microneme protein MIC4	*Toxoplasma gondii ME49*	Microneme protein MIC4
NCLIV_028680	64,290	Putative apical membrane antigen 1	*Toxoplasma gondii GT1*	Apical membrane antigen AMA1

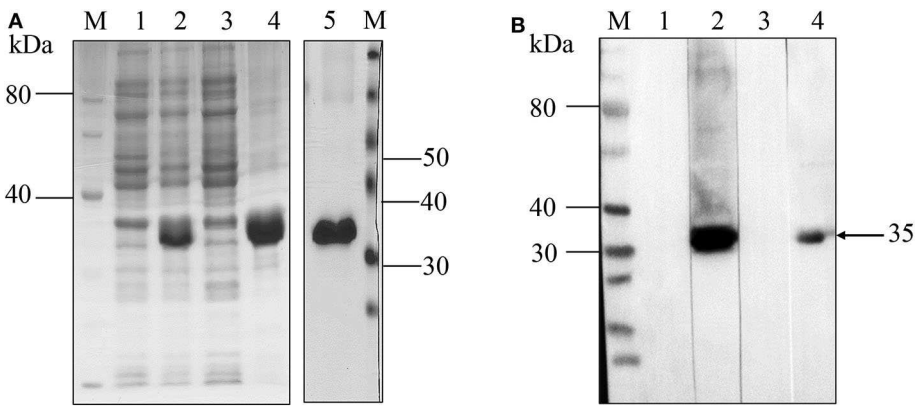

FIGURE 2 | SDS-PAGE and western blot analyses of recombinant NcMIC26 protein. **(A)** Expression and purification of rNcMIC26. Recombinant protein expression patterns were analyzed by SDS-PAGE and visualized by Coomassie blue staining. M: protein marker; lane 1, uninduced protein; lane 2, induced protein; lane 3, supernatant; lane 4: inclusion bodies; lane 5, recombinant protein purified by Ni-NTA Superflow columns. **(B)** Western blotting was used to confirm the reactogenicity and antigenic specificity of *N. caninum*. M: protein marker; lanes 1–4 loading sample: purified rNcMIC26. The incubated antibodies were as follows: lane 1, non-infected mouse serum (1:400); lane 2, mouse *N. caninum*-positive serum (1:400); lane 3, mouse *T. gondii*-positive serum (1:400); lane 4, mouse anti-His monoclonal antibody (1:500); and HRP-labeled goat anti-mouse IgG (H + L) secondary antibody (1:5,000).

Recombinant NcMIC26 Had No Cross-Reaction With *T. gondii*-Positive Serum

The coding sequence of the 744 bp truncated NcMIC26 gene encoding a target protein of 248 amino acids was inserted into the bacterial expression vector pET-28a and expressed as a His fusion protein in *E. coli*, with a predicted molecular mass of 35 kDa. The recombinant NcMIC26 was expressed mainly as inclusion bodies (**Figure 2A**). The molecular mass of the purified recombinant protein was 35 kDa, as expected. Western blot analysis showed that rNcMIC26 reacted strongly with *N. caninum*-positive serum and had no reaction with *T. gondii*-positive serum (**Figure 2B**). This result indicates that the recombinant protein rNcMIC26 has strong reactivity and that rNcMIC26 does not react with *T. gondii*-positive serum, possibly making it a candidate diagnostic antigen for neosporosis.

NcMIC26 Localized on Micronemes

To localize NcMIC26, NcMIC26 was fused with a triple hemagglutinin (3 × HA) epitope tag in the C-terminus by single homologous recombination (**Figure 3A**). NcMIC26 subcellular localization was visualized by immunofluorescent staining using

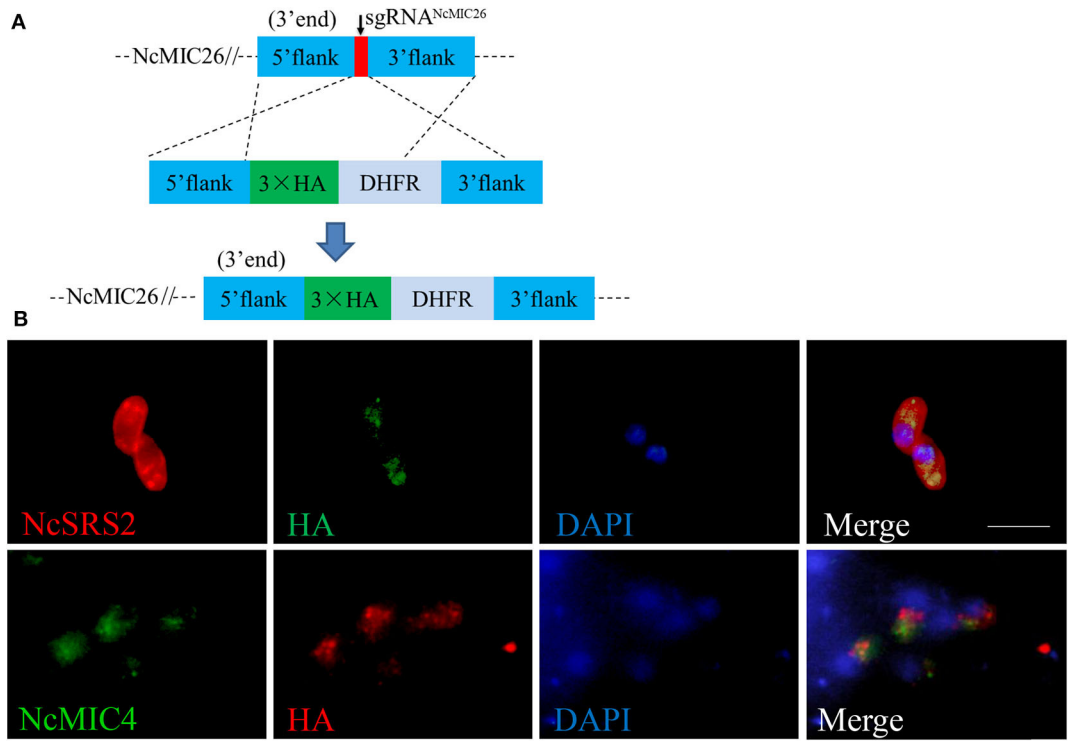

FIGURE 3 | Characterization of NcMIC26 in *N. caninum*. **(A)** Schematic diagram showing NcMIC26 with an HA tag at its endogenous locus. **(B)** Localization of NcMIC26. NcMIC26, stained with mouse anti-HA antibody, was distributed in the microneme of NcMIC26-HA parasites. NcSRS2 was used as a marker to indicate the outlines of parasites; NcMIC4 was used as a marker to indicate the microneme of parasites. Visualized with Cy3-conjugated goat-anti-rabbit IgG (H + L) and FITC-conjugated goat-antimouse IgG (H + L), and nuclear DNA was stained with DAPI (blue). Scale bar, 5 μm.

a mouse anti-HA monoclonal antibody (1:50, Sigma-Aldrich) and rabbit anti-NcSRS2 polyclonal antibody (1:500) (20).

The anti-HA-labeled parasites showed that NcMIC26 was distributed in the micronemes of parasites (**Figure 3B**) and partially colocalized with another microneme marker, NcMIC4 (22), suggesting that NcMIC26 is a microneme protein in *N. caninum*.

Diagnosis of *N. caninum* Infection in Cattle by ELISA With rNcMIC26

Ninety-eight samples of *N. caninum*-positive and 39 samples of *N. caninum*-negative bovine serum were defined by IFAT and evaluated by indirect ELISA based on the rNcMIC26 and rSRS2 antigens; OD values were listed in **Table S2**. The sensitivity and specificity of the ELISA were evaluated. The results are shown in **Table 2**. Compared with that of IFAT, the positive coincidence rate (sensitivity) of the ELISA based on rNcMIC26 (cutoff = 0.150) was 76.53% (75/98), and the negative coincidence rate (specificity) was 84.62% (33/39), while those of the ELISA based on rNcSRS2 (cutoff = 0.178) were 66.33% (65/98) and 92.31% (36/39), respectively. The sensitivity of the ELISA based on NcMIC26 was better than that of the ELISA based on NcSRS2. In addition, compared with NcSRS2 (kappa = 0.476), the NcMIC26-based ELISA test (kappa = 0.540) was more in agreement with the IFAT test through the calculation of the kappa value.

TABLE 2 | Comparison between the ELISA based on rNcMIC26t or rSRS2 and IFAT coincidence rate.

Antigen	Positive coincidence rate	Negative coincidence rate	Kappa value
NcSRS2	66.33%(65/98)	92.31%(36/39)	0.476
NcMIC26	76.53%(75/98)	84.62%(33/39)	0.540

TABLE 3 | Comparison between the ELISA based on rNcMIC26t and rSRS2 for the diagnosis of neosporosis in cattle.

rNcSRS2	rNcMIC26		
	Positive No.	Negative No.	Total
Positive No.	51	17	68
Negative No.	30	39	69
Total	81	56	137

Afterward, a total of 137 serum samples were analyzed statistically. As shown in **Table 3**, the diagnosis results for bovine serum were not consistent. It is interesting to note that of the

98 *N. caninum*-positive bovine serum samples, 17 samples were confirmed as positive only by the ELISA based on rNcSRS2, while they were confirmed as negative by the ELISA based on rNcMIC26, and 30 samples were confirmed as positive by the ELISA based on rNcMIC26, while they were confirmed as negative by the ELISA based on rNcSRS2.

DISCUSSION

N. caninum is a recently recognized protozoan parasite. Until 1988 (23), it was misdiagnosed as *T. gondii*. It is structurally, antigenically, and molecularly related to *T. gondii*, but these organisms are biologically distinct. One of the parameters to be evaluated is diagnostic specificity, that is, the proportion of test negatives among all animals that are true negative. Cross-reactivities among *N. caninum* and *T. gondii* have been reported (24). An increasing number of proteins have been identified as cross-antigens between the two closely related parasites, for example, MIC3 and AMA1 (25), affecting the diagnostic specificity of serological tests. Therefore, it is urgent to screen new dominant antigens with improved diagnostic abilities and to establish more sensitive and specific serological diagnostic methods.

In this study, a new diagnostic antigen, NcMIC26, from *N. caninum* tachyzoite ESA was screened. This antigen is composed of a Von Willebrand figure type A (vWA) domain and five thrombospondin type 1 (TSP-1) repeat regions. Since its identification, the vWA domain has drawn incredible interest because of its far-reaching effects and its association in a wide assortment of vital cellular functions. In *T. gondii*, the VWA domain likely intervenes in the protein–protein interaction of these proteins with their binding partners, which plays a pivotal part in cell adhesion and intrusion by interceding gliding motility (26). Within the TSP repeat region, several motifs are present that have been implicated in cell binding (27). For most MICs, secretion is started *in vitro* before parasites initiate egress from the host cells. Sera from actually infected cattle recognized an overwhelming protein band with an atomic mass indistinguishable from that of NcMIC26 in *N. caninum* ESA. However, it is possible that there are some proteins from the lysis of parasites in the detected proteins. To avoid this, we have selected the proteins from the three major secretory organelles (microneme, rhoptry, and dense granule) and detected its secretion ability in the following screening process. Finally, NcMIC26, which contains signal peptide and a transmembrane region, was confirmed as a secreted protein by secretion assays (data were not shown). In addition to the affirmation that the bovine antiserum recognized rNcMIC26, the results of the current study suggest that the bovine antisera against the whole parasite recognized NcMIC26 as an immunodominant antigen.

To assess whether recombinant NcMIC26 can be an appropriate antigen for the diagnosis of *N. caninum* disease in cattle, purified recombinant NcMIC26 was assessed in an ELISA. IFAT and the rNcSRS2-based ELISA were used as the comparison test. IFATs are based on intaglio tachyzoites and are respected, among others, as reference serological tests ("gold-standard tests") (28). NcSRS2 is an immunodominant surface protein that is displayed within the bradyzoites and tachyzoites of *N. caninum* (29), and empowers specific serological diagnosis of neosporosis. These results demonstrated that the recombinant NcMIC26 expressed in *E. coli* ought to be a valuable diagnostic reagent for the detection of antibodies to *N. caninum* in cattle. Moreover, our detection information reflects a substantial discrepancy in the overall serum assessment determined by ELISA based on NcSRS2 or NcMIC26. The sensitivity of the ELISA based on NcMIC26 was better than that of the ELISA based on NcSRS2, and a considerable portion of *N. caninum*-positive serum can recognize only one antigen (only NcSRS2 or only NcMIC26). Using single antigens in ELISAs is insufficient to examine simultaneously the antibody response against different antigens to which a host is differentially exposed depending on the stage of infection (i.e., the acute or chronic stage). Our data indicated that the ELISA test utilizing NcMIC26 could be used as a supplementary antigen for missed detection by NcSRS2 to improve *N. caninum* diagnosis, while further study should focus on using both antigens in the same ELISA to prove the advantage. On the other hand, compared with that of IFAT, the positive coincidence rate of the ELISA based on rNcSRS2 (66.33%) in our research may be lower than that in other investigations (30). Considering that our serum samples are from cattle that were naturally infected with *N. caninum*, the stage of infection with *N. caninum* was inconsistent, and the antibody titers may be diverse.

CONCLUSION

In this study, we screened a new microneme protein 26 (NcMIC26) from the excretory secretion antigen (ESA) of *N. caninum* tachyzoites. This study characterized NcMIC26 as an effective microneme protein that is recognized by the sera of *N. caninum*-infected animal hosts. The ELISA specific to NcMIC26 established in this study can aid in a more conclusive determination of *N. caninum* infection in cattle. Ensuing studies will be vital to extending the detection affectability of the assay, and more importantly, it can also be utilized as a supplementary antigen for missed discovery by SRS2. The combination of these two antigens may be considered to obtain more accurate detection information in clinical diagnosis.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JL conceived the project. XW performed the experiments and drafted the manuscript. JL and QL participated in the design of the study and helped to draft the manuscript. XW, JY, and XS participated in the interpretation of the data. All authors read

and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00357/full#supplementary-material

Table S1 | Primers of constructed plasmids pNc_Cas9CRISPR::sgNcMIC26 and pLIC-HA-DHFR-NcMIC26.

Table S2 | The data of tested samples for indirect ELISA based on the rNcMIC26 and rNcSRS2.

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A 10-Year Surveillance of *Eimeria* spp. in Cattle and Buffaloes in a Mediterranean Area

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Coccidiosis due to *Eimeria* spp. are widespread parasitic infections in cattle and water buffaloes and may impair health, welfare, and production of these livestock species. The aims of this study were (i) to investigate the prevalence and seasonal dynamics of eimeriosis and (ii) to characterize the *Eimeria* species in large ruminants in a Mediterranean area, in order to plan effective control strategies. Parasitological data were obtained from a 10-year surveillance program (2010–2019) on 3,631 farms (2,089 buffalo and 1,542 cattle farms) sampled in central and southern Italy. Pooled fecal samples were analyzed using the FLOTAC technique with an analytic sensitivity of 2 oocysts per gram of feces (OPG) utilizing a saturated sodium chloride flotation solution (specific gravity = 1.200). *Eimeria* species identification was performed by morphometric analysis after a one week incubation of oocysts in a 2.5% potassium dichromate solution. The results showed high prevalence of *Eimeria* (up to 100%) in both cattle and buffaloes in the 10 years of surveillance, even if a slight reduction was reported in the last three years. The overall prevalence of eimeriosis was 91.7% (95% confidence interval, 95% CI = 90.2–93.1) in cattle farms and 81.5% (95% CI = 79.8–83.1) in water buffalo farms. The mean OPG value was 66.8 (min = 2; max = 8,065) in cattle and 55.9 (min = 2; max = 15,415) in water buffaloes, but this difference was not statistically significant ($p > 0.05$). In total, nine species of *Eimeria* were found in cattle the most prevalent being *Eimeria bovis*, *E. ellipsoidalis*, *E. cylindrica*, and *E. zuernii*, whereas in water buffaloes eight species of *Eimeria* were found, the most prevalent being *E. ellipsoidalis*, *E. auburnensis*, *E. bovis*, and *E. zuernii*. Mixed infections were common in both ruminant species. The seasonal pattern showed a higher prevalence of eimeriosis in cattle in spring (86.9%) whereas in buffalo farms the prevalence was higher in winter (82.3%) and summer (82.4%). In conclusion, the 10-year surveillance program indicates that eimeriosis is common in cattle and water buffaloes and therefore continuous effective control strategies are needed.

Keywords: eimeriosis, seasonal dynamics, cattle, buffaloes, Mediterranean area

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INTRODUCTION

Coccidiosis due to *Eimeria* spp. are widespread parasitic infections in cattle and water buffaloes and may impair health, welfare, and production of these livestock species (1–3). Animals become infected by the horizontal route, ingesting sporulated oocysts from contaminated feed, water, or pasture or by licking contaminated hair coat (1, 3, 4). Outbreaks in cattle and water buffaloes are associated with several factors, including the species of *Eimeria*, the age of the animals, immunological status of hosts, the dose of the oocysts ingested, and farm management and environmental factors (5–7).

More than 20 *Eimeria* species are described in cattle (8), and among them, 12 species can affect also water buffaloes (*Bubalus bubalis*) (9, 10) although coccidia are usually host-specific parasites. *E. zuernii*, *E. bovis*, and *E. auburnensis* are the most pathogenic species in both hosts worldwide (11, 12), while *E. bareillyi* is a pathogenic species specific only for water buffaloes (13).

Usually adult animals are asymptomatic, although they can be a reservoir for younger ones (14, 15), whereas calves can show gastrointestinal (GI) signs, such as diarrhea, dysentery, dehydration, debilitation, and even death (5, 8).

Compared with cattle, there is limited scientific knowledge about the health of water buffaloes so updated data on parasitic infections (as eimeriosis) is an interesting challenge in this species where knowledge regarding the health consequences of the most common pathologies as well as their economic impact on the entire dairy food chain are still almost rare (16).

Indeed, considering the health and welfare implications, as well as the economic losses due to *Eimeria* infections in ruminant livestock, the knowledge of their geographical distribution, prevalence, and intensity of infection is important to understand the dynamic of infection in relation to biotic (such as age) and abiotic (such as seasonality) factors (6) especially in areas where dairy cattle and water buffalo farms coexist and play a major role for the economy of the region (16). The published studies on eimeriosis in large ruminants in Italy are few and focused mainly on treatment (17–19), while the epidemiological data in Europe are scarce, not updated, and focused only on cattle (11, 20–23).

For these reasons, the aims of this study were (i) to investigate the prevalence and seasonal dynamics of eimeriosis and (ii) to speciate the *Eimeria* in large ruminants in a Mediterranean area, in order to plan effective control strategies.

MATERIALS AND METHODS

Study Area and Design

The study was conducted in three Italian regions: Lazio (latitude = 41°53′35″N; longitude = 12°28′58″E) in the Center, Campania (latitude = 40°49′34″N; longitude = 14°15′23″E) and Basilicata (latitude = 40°38′21″N; longitude = 15°48′19″E) in the South. The study area extends over 40,898 km^2 from the Apennines to the Tyrrhenian Sea where cattle and water buffaloes are bred. The entire area is characterized by high heterogeneity with hills and mountains inland and lowlands mainly near the coast. This area is characterized by mild and wet autumns/winters with an average monthly temperature of 9°C and hot and dry springs/summers with an average monthly temperature of 22°C (24).

Parasitological data were obtained by the Regional Centre for Monitoring of Parasitosis (CREMOPAR, Campania Region, Southern Italy) from a 10-year program (2010–2019) of active and passive surveillance on 3,631 farms (cattle and water buffalo farms) (**Figure 1**). Data related to cattle farms in the Lazio region and water buffalo farms in the Basilicata region were fragmented, so they were not included in the study. Moreover, analysis of yearly prevalence and seasonal dynamics of cattle and buffalo coccidiosis was performed only in Campania region, because full data were available through all the years of this study, due to the continuous monitoring service offered by the Department of Agriculture of the Campania Region, through the activities of CREMOPAR.

Farm Management

Cattle Farms

Cattle (*Bos indicus* and *B. taurus*) are the most common world widespread species of large ruminant livestock. Cattle are raised in diverse production systems ranging from capital-intensive, specialized beef and dairy grass-based and feedlot systems (25).

In the study area, cattle are raised for meat and/or milk production. The dairy farms are characterized by an intensive farming system, with suitable buildings and modern equipment to guarantee animal welfare, in order to maximize the production (26). On the other hand, the meat production is mainly characterized by an extensive farming system, with daily grazing and sheltering in part-time housing. This system allows the animals to graze on poor soils with minimal vegetation. In the study area, the two productive realities coexist: dairy farms are spread in the plain and in the foothills area, while the beef cattle are on grazing and marginal land. The Italian cattle population amounts to more than 5 million onto 145,363 farms (**Figure 2A**). The numbers of cattle farms in Campania and Basilicata represent 7.3% and 1.9% of the Italian farms, respectively (National Data Bank—NDB at 31th December 2019).

Buffalo Farms

Water buffalo (*B. bubalis*) farming is important for the economy of several countries, including Brazil, China, India, Vietnam, and Italy. Mozzarella cheese manufacturing from milk of water buffalo is third-ranked in sales volume in Italy (27). The modern intensive water buffalo breeding is likely to replace the cattle breeds and has almost completely replaced the traditional free-range/semi-free-range buffalo farming (21, 28). Currently, the buffalo management is characterized by technologically advanced and automatic systems (e.g., milking robots, automatic manure cleaning, the use of the pedometer for individual measurements of physiological/production parameters, etc.). The southern provinces of Lazio (Latina and Frosinone), the Campania region, and other two southern provinces not included in the study area (Foggia and Isernia) represent the area of buffalo mozzarella cheese with the Protected Designation of Origin (PDO) mark (29). In Italy, there are 2,711 buffalo farms (**Figure 2B**) with a total of 402,796 animals. Lazio and Campania

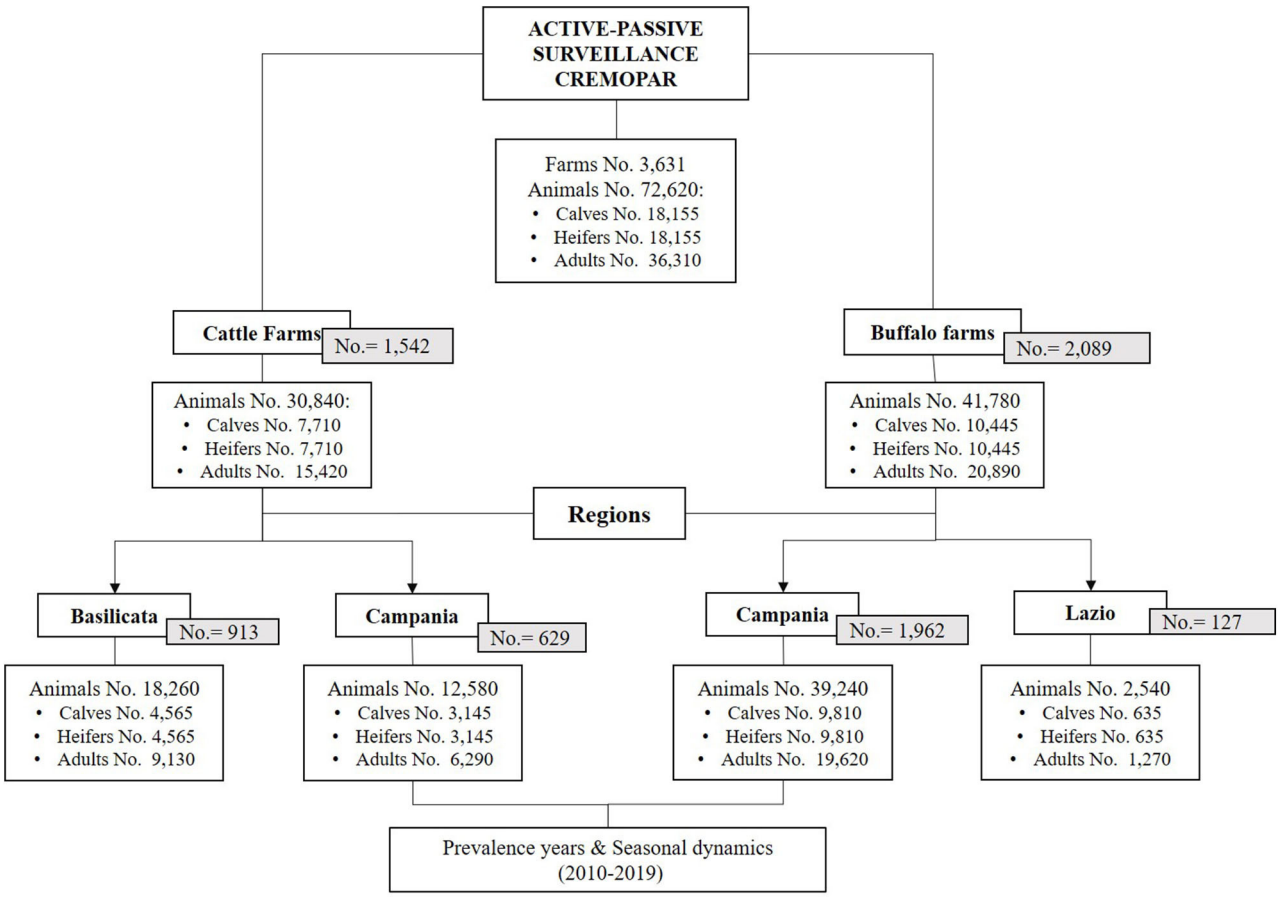

FIGURE 1 | Study setup of the analyzed data from the 10-year surveillance program, with the total number of cattle and water buffalo farms, total number of animals, and age categories in the Italian regions involved (Lazio, Campania, and Basilicata).

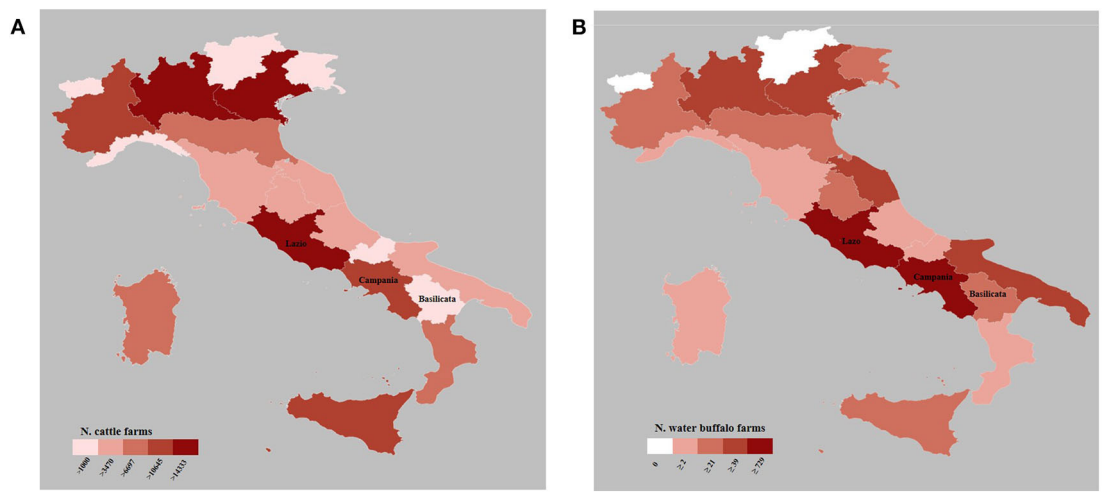

FIGURE 2 | Maps of distribution of cattle **(A)** and buffalo **(B)** farms in Italy at 31th December 2019 (data by National Data Bank).

are the regions with the highest percentage of the total buffalo farms in Italy with 26.9% and 48.8%, respectively (NDB at 31th December 2019).

Copromicroscopic Analysis

A total of 72,620 fecal samples were collected directly from the rectum of animals involved in the study. In each farm,

individual fecal samples (at least 20 g) from 20 animals were collected according to three age groups: 5 calves (0–6 months), 5 heifers (7–12 months) and 10 adults (>12 months). The collected samples were stored by *vacuum* packaging (30) and sent to the laboratories of CREMOPAR. In the laboratory for each farm, 4 pools of feces (one for calves, one for heifers, and two for adults) were prepared, taking 5 g of each individual fecal sample (31). Pooled samples were analyzed by the FLOTAC technique with an analytic sensitivity of 2 OPG, using a sodium chloride flotation solution (specific gravity = 1,200) (32).

In order to sporulate the oocysts and identify the *Eimeria* species, the fecal samples from each positive farm (OPG ≥50) were pooled into one sample (at least 10 g), diluted 1:10 with a 2.5% potassium dichromate solution and incubated in a container at 26–28°C for one week, oxygenating the samples several times a day (33). The *Eimeria* species were identified using the morphometric keys of Eckert et al. (34) and de Noronha et al. (33).

Statistical Analysis

Chi-square (χ^2-test) was employed to verify the association between prevalence and age group of animals and between prevalence of different *Eimeria* species and regions for both hosts. One-way ANOVA test was performed to detect OPG variability between seasons through the years. Difference was considered significant at $P < 0.05$. These statistical analyses were performed with SPSS 23.0 software (IBM, Armonk, NY, USA).

RESULTS

Prevalence of Eimeriosis

Eimeria spp. was found in both cattle and water buffaloes showing a prevalence of 91.7% (95% confidence interval, 95% CI = 90.2–93.1) in cattle farms and 81.5% (95% CI = 79.8–83.1) in water buffalo farms with statistically significant difference ($P < 0.05$). In buffaloes from Lazio, the prevalence was higher than in the Campania region with a statistically significant difference ($P < 0.05$). Regarding OPG, the overall mean value was 66.8 in cattle and 55.9 in water buffaloes, but this difference was not statistically significant ($P > 0.05$). These results were represented in **Figures 3A,B**. The highest prevalence rate and OPG mean values were recorded in young animals (**Table 1**). The one-way ANOVA *test* showed that calves had OPG values significantly higher ($P < 0.05$) in both cattle and buffalo farms.

Yearly Prevalence and Seasonal Dynamics of Infection

Yearly prevalence of positive farms to *Eimeria* infection showed a mean of 86.4% in cattle farms and 82.1% in water buffalo farms. A higher coccidiosis prevalence (100%) was reported in cattle farms from 2012 to 2013, in water buffalo farms from 2012 to 2014. Despite the high prevalence of eimeriosis registered every year, a trend of decrease was recorded in the last three years (from 2017 to 2019) in both hosts.

The general pattern of the excreted mean OPG was very irregular in both hosts. From 2010 to 2013, the values recorded in cattle and water buffaloes were similar, while the highest OPG values were reported in cattle in 2017 and in water buffaloes in 2016.

Although the annual mean prevalence was highest in spring (86.9%) in cattle farms while in water buffalo farms was highest in summer (82.4%) and winter (82.3%), no statistically significant differences ($P > 0.05$) between seasons were found in either hosts.

Identification of *Eimeria* Species

Nine species of *Eimeria* were found in cattle and eight in water buffaloes (**Table 2**). *E. bovis* and *E. zuernii*, the most pathogenic species in cattle, were present in both hosts and in all the three studied regions. *E. bareillyi*, host-specific and pathogenic for buffalo, was found in Lazio and Campania regions with a prevalence of 13.0 and 11.0%, respectively. Mixed infections were common in both livestock species; in particular, 71.2% of cattle and 39.4% of water buffalo farms were infected with more than one *Eimeria* species. In cattle, the prevalence of *E. subspherica*, *E. zuernii*, *E. bovis*, *E. canadensis*, and *E. alabamensis* was higher ($P < 0.05$) in Basilicata than in the Campania region.

DISCUSSION

The 10-year surveillance program indicates that eimeriosis is common (up to 100%) in cattle and water buffaloes in the Mediterranean area studied as in different parts of the world (5, 7, 8, 11, 13, 15, 35, 36). The overall prevalence of *Eimeria* spp. was higher in cattle farms (91.7%) than in water buffalo farms (81.5%). These findings could be explained by the best management practices of modern intensive water buffalo breeding. In particular, the mean coccidiosis prevalence in cattle farms reported in the Campania region in our study (88.3%) was lower than the value of 100% detected in a previous study performed in extensive farms in southern Italy (20). For water buffalo farms, the mean prevalence (80.6%), in the decade 2010–2019, showed a small reduction compared to 97.7% reported in the previous decade (2000–2009) in the Campania region (21, 37). Therefore, these results are in agreement with the earlier findings of the 10-year analysis, showing that the epidemiology of *Eimeria* spp. in this study area has changed over time with a slight reduction in the last three years. This decrease may be due to a control plan implemented by CREMOPAR which started in 2014 through the Rural Development Programme (38) of Campania Region aimed to promote regular and accurate parasitological diagnosis, treatment strategy, and dissemination of best practices of management to cattle and water buffalo farmers. Nonetheless, *Eimeria* is still widespread in the cattle and water buffalo farms.

The mean OPG value was 66.8 (min = 2; max = 8,065) in cattle and 55.9 (min = 2; max = 15,415) in water buffaloes, but this difference was not statistically significant ($P > 0.05$). The mean OPG levels were statistically higher in calves (174.3) than in adult animals (43.2), in both livestock hosts, in agreement with other studies performed in cattle in different countries as

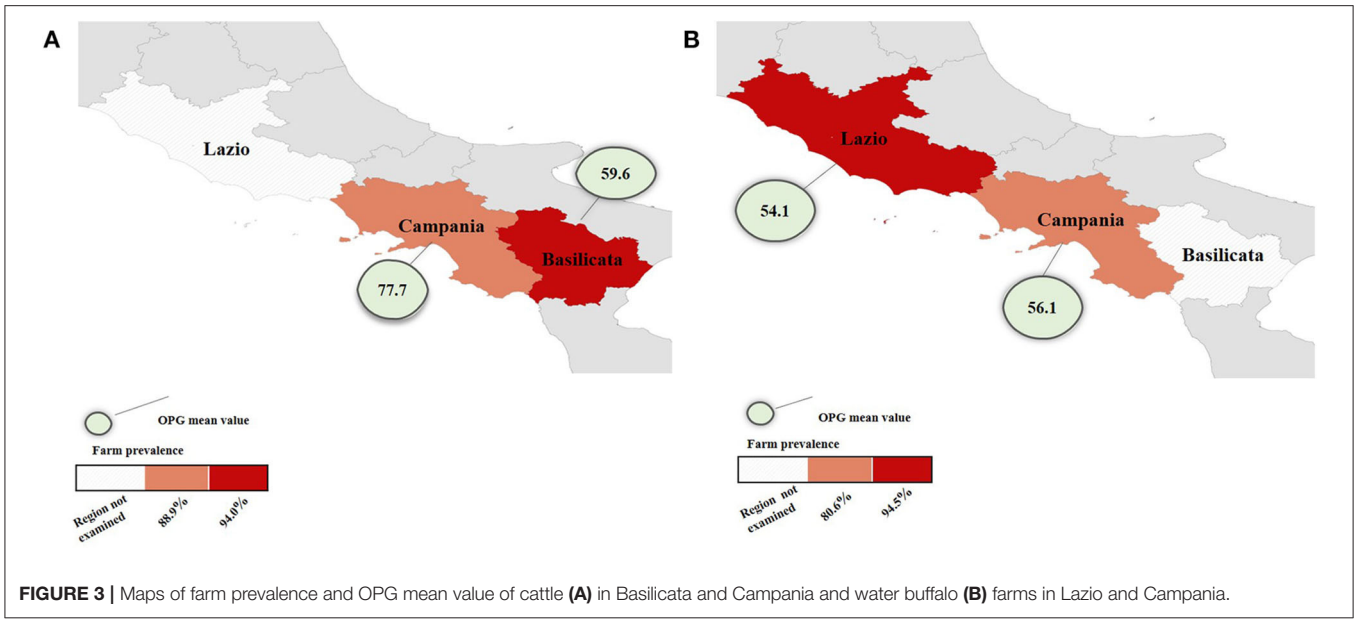

FIGURE 3 | Maps of farm prevalence and OPG mean value of cattle **(A)** in Basilicata and Campania and water buffalo **(B)** farms in Lazio and Campania.

TABLE 1 | Farm prevalence (95% CI) of *Eimeria* spp., age-group mean OPG, minimum, and maximum OPG values, in cattle farms in Campania and Basilicata regions and in buffalo farms in Lazio and Campania regions.

Region	Host	Farm prevalence% (95% CI)	Oocysts per gram of feces (farm prevalence%)				
			Mean (prevalence%)			Minimum	Maximum
			Calves	Heifers	Adults		
Campania	Cattle	88.9 (86.1–97.1)	167.2 (61.0)	65.6 (67.8)	40.1 (38.8)	2	8,065
Basilicata	Cattle	94.0 (92.2–95.4)	188.3 (89.9)	47.4 (87.7)	45.6 (39.8)	2	8,005
Lazio	Water buffalo	94.5 (88.6–97.6)	145.0 (83.3)	46.8 (83.3)	17.0 (74.2)	2	2,250
Campania	Water buffalo	80.6 (78.9–82.3)	256.1 (80.3)	39.7 (77.7)	24.8 (51.8)	2	15,415

Pakistan (39), Germany (40), Kenya (5), and Mexico (7) and in water buffaloes in Brazil (33) and in Pakistan (41). The results of seasonality showed there were no significant differences between the seasons.

Some authors found statistically significant differences between seasons and prevalence in animals (1, 7, 8, 35, 41, 42), but in the Mediterranean area the large ruminant farming system is mainly intensive and so the presence of *Eimeria* might not be influenced by the weather or by grazing, but rather by overcrowding and herd management (e.g., hygiene of pens).

The most prevalent species of *Eimeria* found in this study were *E. bovis* (67.9%), *E. ellipsoidalis* (39.1%), *E. cylindrica* (31.8%), and *E. zuernii* (30.3%) in cattle. These species were widespread also in other countries (8, 12, 36, 43, 44), while some species, such as *E. pellita*, *E. bukidonensis*, and *E. brasiliensis* (7, 8, 12, 43, 44), were not found in our study. In water buffaloes, *E. ellipsoidalis* was the most prevalent (36.3%) species, followed by *E. auburnensis* (26.7%), *E. bovis* (22.3%), and *E. zuernii* (18.2%); in addition, *E. bareillyi*, the buffalo host-specific species, showed a prevalence of 12.3%. These *Eimeria* species were found also in other countries, such as Netherlands, Egypt, Turkey, Iran, Pakistan, India, and Brazil (13, 35, 45), while *E. cylindrica*, *E. alabamensis*, *E. canadensis*, *E. brasiliensis*, and *E. bukidnonensis* found by several authors (13, 35, 45) were not found in our study. Mixed infections with more than one species were common in cattle and buffalo farms with values of 71.2 and 39.4%, respectively. Of the *Eimeria* species detected in this study, only *E. bovis*, *E. zuernii*, *E. auburnensis*, and *E. bareillyi* are responsible of severe clinical disease due to intestinal lesions with effects on the digestive process and overall homeostasis (46). However, the presence of clinical eimeriosis was not assessed in this study and further research is needed to investigate the effects of different species and OPG level on disease development in cattle and buffaloes.

Eimeria species in cattle and water buffalo are identified only through morphological characteristics, but to date there are no studies showing that species in cattle are genetically identical to the ones in water buffaloes. For this reason, molecular techniques using the 18S ribosomal RNA (rRNA) region can be used, not only to identify *Eimeria* species but also to study intra- and inter-genetic variations in cattle and water buffalo species (47, 48). The accurate

TABLE 2 | Prevalence of *Eimeria* species identified in cattle farms in Campania and Basilicata regions and in water buffalo farms in Lazio and Campania regions.

Region	Basilicata	Campania		Lazio
Host	**Cattle**	**Cattle**	**Buffalo**	**Buffalo**
		Prevalence% (95% CI)		
***Eimeria* species**				
*E. subspherica**	26.2 (20.2 – 33.1)	12.5 (7.1 – 20.8)	18.7 (13.1 – 25.9)	17.2 (10.6–26.4)
*E. zuernii**	40.3 (33.4 – 47.7)	20.2 (13.2 – 29.4)	18.1 (12.5 – 25 – 2)	18.2 (11.4–27.5)
*E. ellipsoidalis**	43.5 (36.4 – 50.8)	34.6 (25.7 – 44.7)	36.1 (28.7 – 44.3)	36.4 (27.1–46.7)
*E. cylindrica**	36.1 (29.4 – 43.4)	26.9 (18.9 – 36.7)	0.0	0.0
*E. alabamensis**	6.3 (3.4 – 11.0)	1.0 (0.1 – 6.0)	0.0	0.0
*E. bovis**	78.0 (71.3 – 83.5)	57.7 (47.6 – 67.2)	21.3 (15.3 – 28.7)	23.2 (15.6 – 3 3.0)
*E. canadensis**	12.0 (7.9 – 17.7)	2.9 (0.8 – 8.8)	0.0	0.0
*E. wyomingensis**	7.9 (4.6 – 12.9)	7.7 (3.6 – 15.0)	9.7 (5.7 – 15.7)	0.0
*E. auburnensis**	8.9 (5.4 – 14.1)	10.6 (5.6 – 18.5)	27.1 (20.4 – 34.9)	26.3 (18.2–36.2)
*E. brasiliensis**	0.0	0.0	0.0	0.0
E. pellita	0.0	0.0	3.2 (1.2 – 7.8)	0.0
*E. bukidnonensis**	0.0	0.0	0.0	0.0
E. bareillyi	–	–	11.6 (7.2 – 18.0)	13.0 (10.8 – 15.9)

**Eimeria species common to cattle and buffalo.*

identification of *Eimeria* species has important implications for disease control (49), selection of treatment strategies [e.g., metaphylactic treatments; (50)], and identification of alternative therapeutic approaches [e.g., ozone and intestinal microbiome; (51, 52)].

Metaphylactic treatments with toltrazuril was very useful against *Eimeria* infections in cattle (18, 50, 53), as well as in water buffaloes (19), showing improved performances in animals (e.g., faster body weight gain, positive influence on the average age at the first birth, increased overall percentage of pregnancies). Moreover, a reduction in oocyst excretion was demonstrated, with particular reference to the two species considered to be mainly responsible for clinical coccidiosis (*E. zuernii* and *E. bovis*) (18). Therefore, the metaphylactic approach should also contribute to the reduction in environmental contamination with oocysts, limiting the infection pressure (18, 54). However, the efficacy of toltrazuril could be increasingly reduced by the development of *Eimeria* resistance in ruminants (55). Thus, new low-cost and eco-friendly anti-*Eimeria* strategies are urgently required. Alternative therapeutic approaches based on ozone in ruminants (51) could be useful to control *Eimeria* infections as demonstrated in poultry (56). Moreover, recent studies have highlighted the complex network of interactions occurring between protozoa and the gut commensal flora, showing the potential contribution of the intestinal microbiome in the control of parasitic infections (52).

In conclusion, the findings obtained showed that the coccidiosis is a persistent and complex problem, so a combination of good management practice, affordable diagnostic techniques, and strategic treatments (traditional and/or alternative) could be useful to plan an effective control of *Eimeria* infections in large ruminants.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This animal study was reviewed and approved by Ethic Committee of the Department of Veterinary Medicine and Animal Production, University of Napoli Federico II. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

LA, GS, GB, GC, and LR contribute to the conception and design of the study. MEM and AB organized the database. MEM, AB, MPM, and LR wrote the manuscript. MEM and MPM performed the statistical analysis and GIS. All authors contributed to manuscript revision and read and approved the submitted version.

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GENERAL COMMENTARY
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Commentary: Dogs and the classic route of Guinea Worm transmission: an evaluation of copepod ingestion

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A Commentary on

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Dracunculiasis was largely considered a parasitic disease exclusively affecting humans. That is why all the control measures taken, aimed at its global eradication, were exclusively applied to humans. Currently, Guinea worm disease is considered a zoonosis, with dogs being the main reservoir, reaching high rates of infection, thus jeopardizing its eradication. An alternative route of foodborne parasite transmission has been suggested for dogs by means of the ingestion of infected frogs and/or fish. In addition, a recent study carried out in dogs to assess their ability to ingest copepods while drinking has cast doubts on the key role of drinking water in the dracunculiasis epidemiology. As a result, both routes of transmission, waterborne and foodborne, are discussed.

Around since antiquity, Dracunculiasis, or Guinea worm disease, is a parasitic infection known to affect humans (1). According to the World Health Organization, dracunculiasis was the first parasitic disease set for eradication (https://www.who.int/dracunculiasis/eradication/en/). Hosts become infected by drinking water contaminated with infected copepods harboring the infective L3 larvae. Once infected, the adult female inhabits the host's subcutaneous tissue. A blister is formed on the skin of the host and the parasite embryos are released from the female when the ulcer is exposed to water.

Ever since Fedcheko described the *Dracunculus medinensis* life cycle in 1870 (2) and until 2014, only the waterborne route of Guinea worm transmission was considered in the epidemiology of human dracunculiasis. Guinea worm disease has classically been considered an anthroponosis, i.e., an infectious disease affecting exclusively humans. However, dracunculiasis is nowadays considered a zoonosis since several mammals, mainly dogs, act as reservoirs of the disease in several African countries (3).

In 2014, Eberhard and colleagues, in an attempt to give an explanation for the large number of infected dogs compared to the few scattered human cases in Chad (Africa), proposed a foodborne route of transmission, i.e., by eating aquatic paratenic/transport hosts—frogs and fish, respectively—harboring the infective larvae (4). Considering that the great success of the Guinea worm eradication program (from 3.5 million human cases in the 1980s to only 53 in 2020) (5) has been achieved through the implementation of control measures only against waterborne transmission, the foodborne route seems to be only of anecdotal importance, at least in humans (6).

The classic waterborne route was recently evaluated in dogs by Garret et al. (7). In their experiment, groups of dogs were given drinking water containing different concentrations of

copepods of similar size and species to those which can act as intermediate hosts for the infective Guinea worm larvae. The quantity of copepods ingested by the dogs was estimated and the probability of dogs becoming infected with sufficient male and female larvae to establish an infection was determined using field data on the prevalence of this parasite in wild copepods. According to their results, the authors of the study concluded that drinking water may be an unlikely route for dogs (7).

Consequently, the fact that, in accordance with the experiment carried out by Garret and colleagues, the waterborne route of transmission is unlikely for dogs leads to the assumption that the other possible route of acquiring Guinea worm infection, the foodborne route, i.e., by means of eating infected frogs and fish, should be considered to have a higher likelihood in order to explain the high rate of dog dracunculiasis in Chad.

The various likelihoods are discussed below:

I) Likelihood of eating infected frogs. To date, eight infective larvae of *D. medinensis* have been found in the muscles of only five frogs out of 364 studied; that is a 1.37% prevalence (8, 9). Although further studies are required to establish the real approach to frog dracunculiasis prevalence, it will probably be not much higher. The existence of infected copepods in ponds is presumed to be relatively low, and the likelihood of dogs ingesting multiple infected copepods during the 2 to 3 weeks in which copepods are infected with L3 may be low (7). Since frogs and dogs share the same waterborne route of dracunculiasis infection (the ingestion of infected copepods), tadpoles or frogs will have an even lower likelihood of becoming infected (considering their small size compared to dogs). We therefore ask the following: how many frogs have to be eaten by a dog to have the chance of ingesting the infected ones (with male and female larvae) to finally be infected? Considering this low likelihood, the frog route probably does not have any epidemiological importance in the transmission. In this sense, a recent paper by McDonald and colleagues determined that the diet of Chadian dogs consisted basically of human feces and potatoes, peanuts, and rice. Other items, including frogs and fish, are apparently infrequently eaten. The authors consider novel Guinea worm routes of infection as occasional introductions (10). In addition, a study conducted in Chad showed that there were no differences in consumption of aquatic animals, including frogs, between human dracunculiasis cases and controls (11).

II) Likelihood of eating infected fish. Although L3 larvae have not been found in any fish so far, fish are considered possible transport hosts (8). In experimental infections, after ingesting infected copepods, larvae remained infective in fish intestines. L3 larvae were highly susceptible to desiccation and exhibited a short life span in the fish intestinal tract (8). Therefore, for dogs to become infected, those transport fish should be caught, taken to the village, eviscerated, and have their entrails discarded in order for dogs to eat them (mostly the infected guts among all the non-infected ones), and the entire process must take place fast enough to prevent larvae desiccation/death. According to the Carter Center/CDC, dog dracunculiasis cases increase considerably in summer (12). The incubation period of *D. medinensis* is around 1 year, and, consequently, dogs become more frequently infected in summer when temperatures reach up to 43°C in Chad (in the shade). It is not believed that Chadian fishers are worried about preventing entrails, or discarded small fish, from desiccation. There is consequently not a high likelihood of this fish route to be the main cause.

III) Likelihood of drinking water with infected copepods. Dogs drink unsafe (unfiltered) water several times a day every single day of their lives, and this logically continues in large amounts during the summer, precisely when dog infection increases. Dogs are therefore exposed to permanent risk by the water route. The type of food consumed by dogs can vary, but dogs drink water on a daily basis from whatever water source they may find. The lack of any reliable dog surveillance program before 2012 (like the one currently conducted) does not make it possible to establish the exact degree of exceptionality of the dog epidemiology in Chad, where humans, unlike dogs, thanks to the Guinea Worm Eradication Program, have been drinking filtered water—the reason why only sporadic cases have been detected.

According to their findings, Garret and colleagues do not rule out or minimize the waterborne route, but they doubt its epidemiological importance in dog dracunculiasis (7). However, the same authors recognized the limits of their experiment due to, among other factors, the small sample size chosen. Besides, the dogs were kept at 21°C, not precisely at the extreme temperatures in Chad in the peak of the transmission season (40–43°C). Consequently, the drinking habits of dogs could not be comparable. Furthermore, they used copepods from the USA, which may behave differently from those from Chad. However, and more importantly, the authors did not consider that the copepods used in the experiment were non-infected ones. It has been demonstrated that non-infected and infected copepods with helminth larvae, not only with *D. medinensis*, display a different behavior that positively affects parasite transmission (13–16). Therefore, Garret and colleagues' conclusions could be entirely accepted as well as reasonably discussed based on the data currently known.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Dairy Calves in Uruguay Are Reservoirs of Zoonotic Subtypes of *Cryptosporidium parvum* and Pose a Potential Risk of Surface Water Contamination

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Cryptosporidium parvum, a major cause of diarrhea in calves, is of concern given its zoonotic potential. Numerous outbreaks of human cryptosporidiosis caused by *C. parvum* genetic subtypes are reported yearly worldwide, with livestock or water being frequently identified sources of infection. Although cryptosporidiosis has been reported from human patients in Uruguay, particularly children, epidemiologic information is scant and the role of cattle as reservoirs of zoonotic subtypes of *C. parvum* has not been explored. In this study, we aimed to (a)-identify *C. parvum* subtypes infecting dairy calves in Uruguay (including potentially zoonotic subtypes), (b)-assess their association with calf diarrhea, (c)-evaluate their spatial clustering, and (d)-assess the distance of infected calves to surface watercourses draining the farmlands and determine whether these watercourses flow into public water treatment plants. Feces of 255 calves that had tested positive for *Cryptosporidium* spp. by antigen ELISA were selected. Samples had been collected from 29 dairy farms in seven Uruguayan departments where dairy farming is concentrated and represented 170 diarrheic and 85 non-diarrheic calves. Selected samples were processed by nested PCRs targeting the 18S rRNA and gp60 genes followed by sequencing to identify *C. parvum* subtypes. Of seven *C. parvum* subtypes detected in 166 calves, five (identified in 143 calves on 28/29 farms) had been identified in humans elsewhere and have zoonotic potential. Subtype IIaA15G2R1 was the most frequent (53.6%; 89/166), followed by IIaA20G1R1 (24.1%; 40/166), IIaA22G1R1 (11.4%; 19/166), IIaA23G1R1 (3.6%; 6/166), IIaA17G2R1 (3%; 5/166), IIaA21G1R1 (2.4%; 4/166), and IIaA16G1R1 (1.8%; 3/166). There were no significant differences in the proportions of diarrheic and non-diarrheic calves infected with any of the *C. parvum* subtypes. Two spatial clusters were detected, one of which overlapped with Uruguay's capital city and its main water treatment plant (Aguas Corrientes), harvesting

surface water to supply ∼1,700,000 people. Infected calves on all farms were within 20–900 m of a natural surface watercourse draining the farmland, 10 of which flowed into six water treatment plants located 9–108 km downstream. Four watercourses flowed downstream into Aguas Corrientes. Calves are reservoirs of zoonotic *C. parvum* subtypes in Uruguay and pose a public health risk.

Keywords: bovine cryptosporidiosis, *Cryptosporidium parvum* zoonotic subtypes, dairy calves, diarrhea, spatial clusters, surface water, Uruguay

INTRODUCTION

Cryptosporidiosis is a global disease caused by protozoa of the genus *Cryptosporidium*. It affects a wide variety of hosts, including humans and ruminants. The predominant species that infect cattle are *C. parvum*, *C. andersoni*, *C. bovis*, and *C. ryanae* (1). *C. parvum* is a major cause of diarrhea in neonate calves, which shed large amounts of highly resistant fecal oocysts that contaminate the environment (2). Moreover, it is a zoonotic pathogen and a leading cause of water- and foodborne diarrheal disease in humans (3). Sources of *Cryptosporidium* infection to humans include contaminated surface water (lakes, rivers), municipal drinking water (as oocysts are largely resistant to chlorination), recreational water (swimming pools, water playgrounds), food, and infected livestock (4–6). Of the numerous outbreaks of human cryptosporidiosis reported annually worldwide (7), many have been linked to cattle as sources of *C. parvum* infection (5, 8, 9).

After fecal-oral transmission, *C. parvum* infects the host enterocytes and undergoes a phase of sexual reproduction, during which the recombination of genes takes place, with the consequent generation of different genetic families and subtypes that, depending on epidemiological conditions, can differ between and within geographical regions (10, 11). It is not possible to identify *Cryptosporidium* to the species level or *C. parvum* subtypes with conventional techniques traditionally used to detect cryptosporidia, such as acid-fast or auramine-phenol stains and immunological assays, such as direct immunofluorescence or ELISA. However, genetic analysis of the 18S ribosomal RNA gene allows for *Cryptosporidium* species identification and analysis of the glycoprotein 60 (gp60) locus allows not only for *C. parvum* species confirmation, but also for further identification to the family and subtype levels (12). This molecular approach has been used in epidemiologic studies to assess geographic segregation and interspecies transmission (8, 9), which have led to a better understanding of cryptosporidiosis in animals and humans. For instance, evidence indicates that most infections in young calves are caused by *C. parvum*, primarily from the IIa family, which is known to contain the most frequently zoonotic subtype worldwide IIaA15G2R1 (13–15), regarded as a hyper-transmissible subtype (13). Thus, molecular techniques have aided in the understanding of the epidemiological patterns and transmission chains of cryptosporidiosis and can ultimately help to delineate prevention and control strategies (16).

Exposure to recreational water (35.1%) and direct contact with cattle (14.6%) were the main sources identified in 444 outbreaks of cryptosporidiosis reported in humans in the USA in 2009–2017 (16). Human cryptosporidiosis has been documented in Uruguay (17, 18), although the sources of infection have not been explored. With ∼12.2 million head of cattle in 2017–2018, and a total human population of 3.53 million, Uruguay is the country with the highest number of cattle per capita worldwide (19). The area allocated to cattle farms accounts for ∼75% of the country's territory (20). Most cattle are raised outdoors in pasture-based farming systems, which causes environmental contamination with feces, exposure of fecal depositions to rainfall, and cattle access to surface natural watercourses. Uruguay's topography is represented by water-rich land and flat plains that sometimes flood. Its humid temperate climate without a dry season (21), annual rainfalls of 700–1,200 mm, along with the dense network of surface natural watercourses (22) averaging 1.4 linear km per km^2 of area, provide favorable conditions for the transmission of waterborne disease agents.

Considering the reservoir potential of cattle and that *Cryptosporidium* spp. is a frequent cause of diarrhea in dairy calves in Uruguay (23), we wondered whether *Cryptosporidium* species and subtypes infecting cattle could pose a potential risk to public health through either direct contact or surface water contamination. Given the current scenario, in this study, we aimed to (a) identify *Cryptosporidium* species and subtypes infecting dairy calves in Uruguay, including potentially zoonotic *C. parvum* subtypes, (b) assess the association of different *C. parvum* subtypes with calf diarrhea, (c) evaluate their spatial clustering, and (d) assess the distance of infected calves to natural surface watercourses draining the farmlands and determine whether these watercourses flow downstream into public water treatment plants, which may indicate a potential risk to public health.

MATERIALS AND METHODS

Samples and Farms

A total of 255 stool samples from dairy calves stored at −20°C at the Instituto Nacional de Investigación Agropecuaria (INIA) veterinary diagnostic laboratory (Plataforma de Investigación en Salud Animal) were selected for this study. All samples were non-randomized, had been collected for another study between January and November 2016, and had tested positive for *Cryptosporidium* spp. antigen using a commercial antigen capture ELISA kit (Pathasure Enteritis-4; Biovet, St. Hyacinthe,

Quebec, Canada) (23), which was an inclusion criterion. Samples represented 170 diarrheic and 85 non-diarrheic dairy calves up to 30 days of age, from 29 commercial dairy farms (farms 1–29) located in seven departments of Uruguay (Colonia, San José, Flores, Soriano, Florida, Canelones and Río Negro). In the original study (23), feces of 552 diarrheic (n: 267, cases) and non-diarrheic (n: 285, controls) neonate dairy calves were sampled. Samples were obtained from commercial farms experiencing spontaneous cases of neonatal diarrhea (convenience sampling). The sample size was calculated using a free online calculator (Epitools, Australia: https://epitools.ausvet.com.au/casecontrolss?page=case-controlSS), considering a power of 80% to detect an association between diarrhea and infection with a given pathogen, a percentage of exposed controls of 5%, and an Odds Ratio (OR) of 2.5 with a 95% confidence level. Of all 552 calves tested by ELISA for *Cryptosporidium* spp. antigen, 265 (48%) resulted positive, 255 of which were available for this study. The sampling protocol was approved by INIA's animal ethics committee for the use of animals in experimentation (CEUA, protocol # 20199).

Additional information was collected from the farms, including herd size (number of milking cows and number of reared calves in 2016), the area (surface in m^2) of the calf-rearing areas, the type of calf housing in the calf-rearing areas (individual vs. collective or group pens, indoors vs. outdoors), the type of floor in the calf-rearing areas, whether feces were removed from the floor of the calf-rearing areas, and the drinking water sources for the calves (**Supplementary Table 1**).

DNA Extraction, PCR Amplification, and Sequencing for *Cryptosporidium* Speciation and Subtyping

DNA was extracted from 150 mg of each of the 255 fecal samples using a commercial kit (Quick DNA Fecal/Soil Microbe Miniprep Kit; Zymo Research, Irvine, CA, USA), following the manufacturer's instructions. A nested PCR protocol targeting the 18S rRNA gene for the detection and speciation of *Cryptosporidium* spp. was performed using the PCR primers 5′-TTCTAGAGCTAATACATGCG-3′ and 5′-CCCATTTCCTTCGAAACAGGA-3′ (∼1,319 bp) and the nested PCR primers 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and 5′-AAGGAGTAAGGAACAACCTCCA-3 (∼834 bp) as previously described (24, 25). Additionally, *C. parvum* speciation and subtyping were performed using a two-step nested PCR protocol targeting a fragment of the gp60 gene using the PCR primers 5′-ATAGTCTCCGCTGTATTC-3′ and 5′-GGAAGGAACGATGTATCT-3′ (∼900 bp), and the nested PCR primers 5′-TCCGCTGTATTCTCAGCC-3′ and 5′-GCAGAGGAACCAGCATC-3′ (∼860 bp) (26), in 166 (74.8%) of the 18S rRNA PCR-positive samples. Samples were selected for speciation and subtyping ensuring that all 29 farms were represented. Amplification reactions for the 18S rRNA and gp60 genes were performed in a volume of 25 μL containing Platinum® PCR SuperMix (Life Technologies, Carlsbad, CA, USA), 200 nM of each primer and 2 μL of target DNA in both PCR and nested PCRs. Reactions were performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Samples were denaturated at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing for 30 s. at 55°C (18S rRNA gene) or 50°C (gp60 gene) and extension for 1 min. at 72°C, with a final extension at 72°C for 7 min. *C. parvum* DNA and ultrapure water were used as positive and negative controls, respectively. Amplified fragments were analyzed by GelRed® (Biotium, Fremont, CA, USA) stained gel electrophoresis.

The obtained amplicons for both the 18S rRNA and gp60 genes were purified using a QIAquick Gel Extraction Kit (Qiagen, Santa Clara, CA, USA) and sequenced using the ABI Prism® Dye Terminator Cycling Sequence kit in an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, USA). DNA sequences were assembled and aligned with CodonCode Aligner version 7.1.2 (CodonCode Corporation, Centerville, MA, USA) and BioEdit Sequence Alignment Editor (27), and compared with homologous sequences available in GenBank using Clustal W (28). *C. parvum* subtypes were identified based on the number of TCA (A), TCG (G), and ACATCA (R) repeats (29).

Statistical Analyses

Comparisons among proportions of diarrheic and non-diarrheic calves infected with each different *C. parvum* subtype were performed with a one-sample *z*-test of proportion (when $n \geq 30$) or binomial test (when $n < 30$) for a significance level of $P < 0.05$ using STATA® version 14.0 (StataCorp, College Station, TX, USA).

For spatial cluster detection, multinomial modeling of the spatial scan statistics was performed to assess the relative risk for clusters of each *C. parvum* subtype detected at the farm level, as described previously (30, 31). Briefly, different sized circular windows are placed randomly over the area of study and the likelihood ratio of the cases (each *C. parvum* subtype) clustered within the window is compared with the expected in the remaining areas as generated by 999 Monte Carlo simulations. Cluster detection was implemented in SaTScan™ version 9.4.4 (Martin Kulldorff, Boston, MA, USA).

Assessment of the Location and Distance Between Infected Calves, Natural Surface Watercourses, and Downstream Surface Water Treatment Plants

The calf-rearing areas of the 29 dairy farms where the infected calves were located were georeferenced and mapped. The elevation and the slope of the farmland, obtained from the digital terrain model of Uruguay (32), were considered to assess the water drainage network for each calf-rearing area. The distance between each calf-rearing area and the nearest surface natural watercourse (streams or rivers) was measured in meters with QGIS software (33), considering the shortest natural water drainage route. Additionally, we assessed whether these natural surface watercourses would flow downstream in the direction of any public water treatment plants harvesting surface water for sanitation and human consumption. The geographic locations of these water plants was obtained from the website of the Uruguayan "Ministerio de Vivienda, Ordenamiento

Territorial y Medio Ambiente" (22), and the distance between these points following the path of the watercourses down to the treatment plants was measured in kilometers using QGIS software.

RESULTS

Cryptosporidium Species and Subtype Identification

Of the 255 samples included in the study, 222 (87.1%) were positive by the nested PCR targeting the 18S rRNA gene, confirming *Cryptosporidium* spp. To conduct *Cryptosporidium* species identification, 60 (27%) of the 222 18S rRNA PCR-positive samples, selected at random and representing all 29 farms, were further sequenced; *C. parvum* was the only species identified in all 60 calves. Based on these results, considering that *C. parvum* is the main species found in calves up to 30 days of age (15), and due to financial constraints, we decided to pursue additional *C. parvum* speciation and subtyping using a nested PCR assay targeting the gp60 gene followed by sequencing. This approach was followed in 166 (74.8%) of the 18S rRNA PCR-positive samples, including the above-mentioned 60 samples identified as *C. parvum* by 18S rRNA amplicon sequencing, and representing all 29 farms. One of seven different *C. parvum* subtypes were identified in all 166 calves (**Table 1**). Nucleotide sequences generated in this study (18S rRNA and gp60 genes) were deposited in GenBank under accession numbers MT010356 through MT010363.

The most frequent subtype in the 166 calves was IIaA15G2R1 (53.6%; 89/166), followed by IIaA20G1R1 (24.1%; 40/166), IIaA22G1R1 (11.4%; 19/166), IIaA23G1R1 (3.6%; 6/166), IIaA17G2R1 (3%; 5/166), IIaA21G1R1 (2.4%; 4/166), and IIaA16G1R1 (1.8%; 3/166). Subtype IIaA15G2R1 was also the most frequent at the farm level (20/29, 69%), and was identified in 100% of the seven departments. Subtype IIaA20G1R1 was identified in 8/29 (27.6%) farms and 3/7 (42.9%) departments (Río Negro, San José and Flores). Subtype IIaA22G1R1 was identified in 6/29 farms (20.7%) in 3/7 departments (Colonia, Río Negro and Florida). The other four subtypes (IIaA23G1R1, IIaA17G2R1, IIaA21G1R1, and IIaA16G1R1) were only identified in individual farms, one in Colonia and three in San José. The latter department was the only one where all seven different *C. parvum* subtypes were found. In seven of the 29 farms (24.1%) more than one subtype of *C. parvum* was found. The combination of subtypes included IIaA15G2R1—IIaA22G1R1 and IIaA15G2R1—IIaA20G1R1 in three farms each, and IIaA15G2R1—IIaA21G1R1 in a single farm. At least one zoonotic subtype was identified in 28 of the 29 (96.6%) farms.

Cryptosporidium parvum Subtypes and Calf Diarrhea

Of the 166 samples subjected to subtyping, 119 were from diarrheic and 47 from non-diarrheic calves. There were no significant differences in the proportions of diarrheic and non-diarrheic calves infected with any of the *C. parvum* subtypes (**Table 2**).

Cryptosporidium parvum Subtypes and Spatial Analysis

The geographic locations of the 29 farms are shown in **Figures 1**, **2**, and the outputs of the spatial analysis are summarized in **Table 3**. Two spatial clusters were identified ($P < 0.0001$). The primary cluster comprised seven farms, located in most of Río Negro, the south of Paysandú, and the north of Soriano (**Figures 1**, **2**). The cluster included a total of 38 infected calves, with the main subtypes being IIaA20G1R1 and IIaA22G1R1 (**Table 3**). The secondary cluster comprised 12 farms located in the departments of San José, Florida, and Canelones, overlapping with the country's largest metropolitan area and capital city, Montevideo (**Figure 2**). The total number of infected calves in this cluster was 67, and the main subtypes identified were IIaA15G2R1, IIaA17G2R1, and IIaA21G1R1.

Location and Distance Between *Cryptosporidium parvum*-Infected Calves, Natural Watercourses, and Downstream Surface Water Treatment Plants

A map showing the natural surface watercourses of the study area and the location of farms with *C. parvum*-positive calves is depicted in **Figure 2**. The distance between the calf-rearing areas on these farms and the nearest watercourse (considering the shortest water drainage route based on the altitude and slope of the terrain), as well as the subtypes detected in each farm, are shown in **Table 4**. The average distance between the calf-rearing areas with *C. parvum*-positive calves (n: 29) and natural surface watercourses was 352 m, with a range of 20–900 m. Ten of these 29 (34.5%) watercourses flowed downstream into six surface water treatment plants located in the departments of Canelones, Flores, Soriano, San José, and Colonia (**Figure 2** and **Table 4**). The average distance between these 10 calf-rearing areas and the respective nearest natural watercourse was 311 m (range: 20–700 m), and the average distance between these points and the closest downstream water treatment plants was 52.95 km (range: 9–108 km). Four of these watercourses, which drained farms in the secondary spatial cluster, flowed downstream into the Santa Lucía River in Canelones, and further down into two water treatment plants (Santa Lucía and Aguas Corrientes) that overlapped with the secondary spatial cluster (**Figure 2** and **Table 4**). The average distance between these four calf-rearing areas and the nearest watercourse was 307.5 m, with a range of 50–550 m; the distances to these two water treatment plants are shown in **Table 4**. At least one zoonotic subtype of *C. parvum* was identified in 28 of the 29 (96.6%) farms, including 9/10 (90%) farms located upstream from water treatment plants (**Table 4**).

Additional Information of the Farms and Calf-Rearing Areas

In the year of sampling (2016) the farms included in the study had herd sizes that ranged from 70 to 1,260 milking cows and raised between 52 and 1,342 calves in calf-rearing areas ranging from 179 to 7,500 m^2. In 24 of the 29 farms (82.8%) calves were raised outdoors on dirt floor either in collective pens (n: 13 farms) or individual housing systems (n: 11 farms), while in

TABLE 1 | Number of < 30-day-old dairy calves infected with different *Cryptosporidium parvum* subtypes in seven departments of Uruguay.

Department	*C. parvum* subtypes							Total
	*IIaA15G2R1	*IIaA20G1R1	IIaA22G1R1	*IIaA23G1R1	*IIaA17G2R1	IIaA21G1R1	*IIaA16G1R1	
Colonia	19	0	10	6	0	0	0	35
San José	48	9	1	0	5	4	3	70
Flores	4	1	0	0	0	0	0	5
Soriano	8	0	0	0	0	0	0	8
Florida	8	0	1	0	0	0	0	9
Canelones	1	0	0	0	0	0	0	1
Río Negro	1	30	7	0	0	0	0	38
Total	89	40	19	6	5	4	3	166

**Subtypes that have been found in humans elsewhere: IIaA15G2R1 (34), IIaA16G1R1 (35, 36), IIaA17G2R1 (35, 37, 38), IIaA20G1R1 (39), and IIaA23G1R1 (40).*

TABLE 2 | Proportion of *Cryptosporidium parvum* subtypes in non-diarrheic and diarrheic calves, and results of the one-sample test of proportion or binomial test.

C. parvum subtype	Consistency of feces				Total		*P*-value
	Non-diarrheic		Diarrheic				
	n	Proportion, 95%CI	*n*	Proportion, 95%CI	*n*	Proportion, 95%CI	
IIaA15G2R1	21	45%, 3.1–58.7	68	57%, 48.1–65.6	89	54%, 46.0–61.0	0.253
IIaA20G1R1	15	32%, 20.4–46.1	25	21%, 14.6–29.2	40	24%, 18.2–31.1	0.203
IIaA22G1R1	2	4%, 1.2–14.2	17	14%, 9.1–21.7	19	11%, 7.5–17.2	0.139
IIaA23G1R1	2	4%, 0.5–14.5	4	3%, 0.9–8.3	6	4%, 1.3–8.0	0.239
IIaA17G2R1	3	6%, 1.3–17.5	2	2%, 0.2–5.9	5	3%, 0.9–7.0	0.166
IIaA21G1R1	3	6%, 1.3–17.5	1	0.8%, 0.02–5.0	4	2%, 0.6–6.0	0.103
IIaA16G1R1	1	2%, 0.05–11.3	2	0.2%, 0.2–6.0	3	2%, 0.3–5.2	0.574
Total	47		119		166		

CI, confidence interval.

4/29 farms (13.8%) calves were raised indoors in individual (n: 2 farms) or collective (n: 2 farms) housing systems with cement (n: 3 farms) or wood floor (n: 1 farm). In all 24 farms raising calves outdoors on dirt floor, calf feces were left on the floor and were not routinely removed from the calf-rearing areas. In the 4 farms raising calves indoors, the cement or wood floor was routinely hosed down, but these facilities did not have a sanitary drainage system for the resulting liquid waste, that overflowed to the adjacent farmland. In all 29 farms (100%) the drinking water source for the calves was untreated underground water. The individual information for each farm and calf-rearing area is summarized in **Supplementary Table 1**.

DISCUSSION

In Uruguay, information on *Cryptosporidium* spp. is scarce, dating since 1986, when the parasite was first detected (18). *Cryptosporidium* spp. was identified as a cause of diarrhea in children in 6.15 and 8.9% of the studied population in two independent studies (17, 18), and later considered an emerging disease in this country (41). More recently, *Cryptosporidium* spp. was recognized as a causative agent of neonatal diarrhea in dairy calves (23), and *C. parvum* was identified in shallow watercourses in industrialized areas of the country (42), the latter being the only description of *C. parvum* species confirmation in Uruguay. However, to the best of our knowledge, there are no descriptions of zoonotic cryptosporidiosis in the country. Considering this background information, we wondered whether calves were reservoirs of zoonotic subtypes of *C. parvum* and could pose a potential risk for public health either through direct contact or surface water contamination.

Cryptosporidium spp. is a major cause of diarrhea in dairy calves in Uruguay. In a case-control study by our group, *Cryptosporidium* spp. antigen was identified by capture ELISA in feces of 189/271 (69.7%) diarrheic and 79/285 (27.7%) non-diarrheic calves from 100% of 30 dairy farms in seven departments where dairy farming is concentrated. Infected calves were six times more likely to be diarrheic than non-infected ones (23). This indicates that cryptosporidiosis is widespread in calves, although the eventual role of cattle as reservoir of zoonotic *Cryptosporidium* strains had not been explored, as the molecular identification of *Cryptosporidium* species and subtypes infecting these calves was not pursued. In this follow-up study, using mostly the same sample set, we showed the wide distribution of *C. parvum* at the calf and dairy farm levels, even with a limited sample size that is unlikely to be representative of the dairy cattle

FIGURE 1 | Map of Uruguay (entire country) showing the geographic distribution of 29 dairy farms with *Cryptosporidium parvum*-positive calves. Numbers indicate the farm identification (1–29). Red lines indicate spatial clusters for different *C. parvum* subtypes, as shown in **Table 4**.

population of the country. The predominance of *C. parvum* is somewhat expected and consistent with the age of the studied calves, as this is the most frequent species in neonate animals (15). In all the farms and departments included in the study, at least one subtype of *C. parvum* was identified, as in other studies carried out in Europe (43–46), Australia (47), Argentina

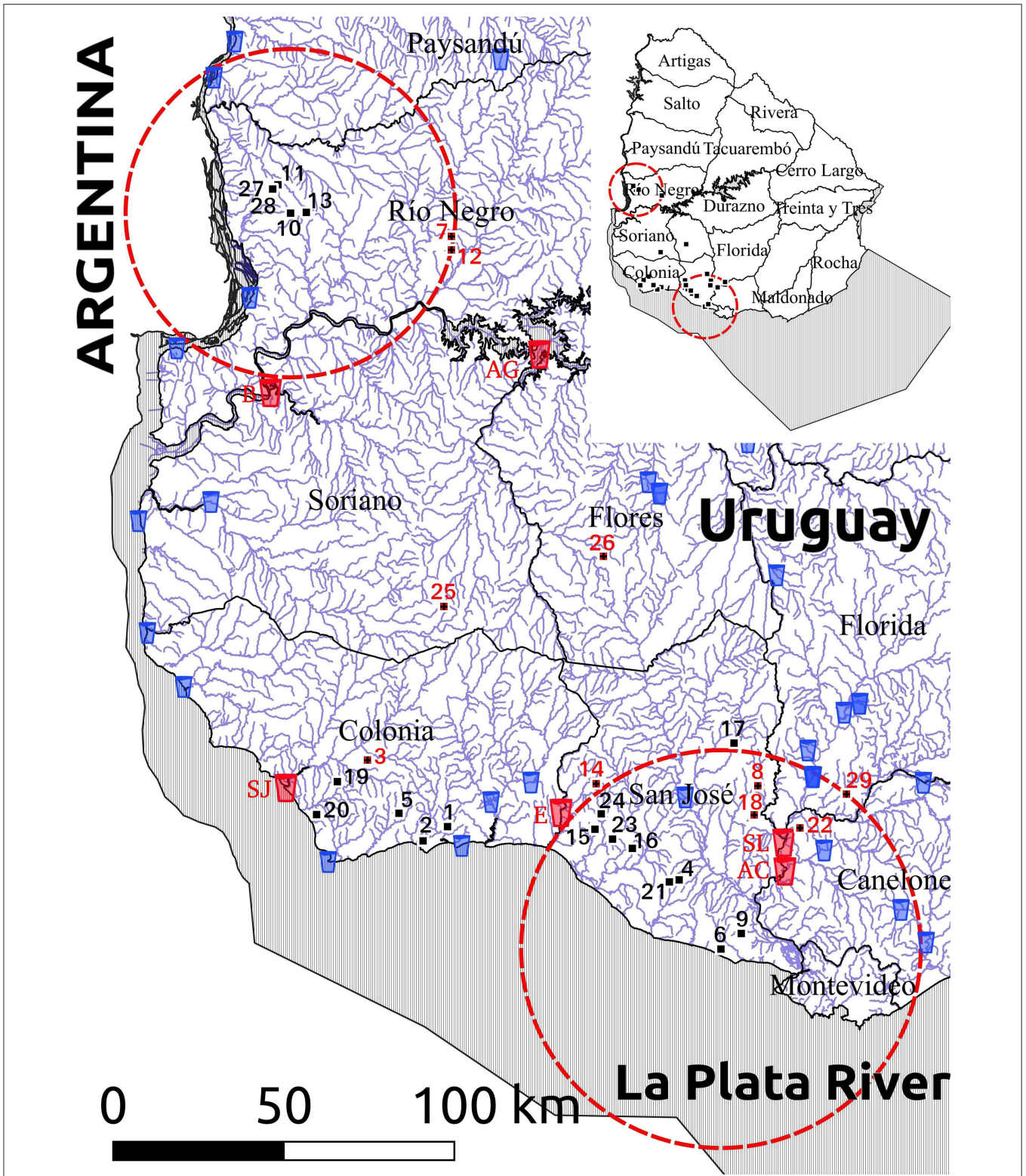

FIGURE 2 | Map of the study area (southwestern Uruguay) showing the geographic distribution of the 29 dairy farms with *Cryptosporidium parvum*-infected calves (numbers), natural surface watercourses (blue lines) and water treatment plants harvesting water for human consumption (glass icons). Ten farms that drain into a watercourse that flows downstream into a water treatment plant are indicated with red numbers, while the six water treatment plants receiving water from these 10 watercourses are highlighted with red glass icons and identified as AC (Aguas Corrientes), AG (Arroyo Grande), B (Bequeló), E (Ecilda), SJ (San Juan), and SL (Santa Lucía). Red lines indicate spatial clusters for different *C. parvum* subtypes, as shown in **Table 4**.

TABLE 3 | Outputs of the spatial analysis for *Cryptosporidium parvum* subtype cluster detection in dairy calves.

Cluster rank	Number of dairy herds in each cluster	Centroid geolocation		Radius (km)	Total number of cases	Subtype	Observed/expected cases for each subtype	RR	LLR	*P*-value
		South	West							
1	7	32.757967	57.931379	48.21	38	IIaA15G2R1	0.049	0.038	48.821332	<0.0001
						IIaA16G1R1	0	0		
						IIaA17G2R1	0	0		
						IIaA20G1R1	3.28	10.11		
						IIaA21G1R1	0	0		
						IIaA22G1R1	1.61	1.96		
						IIaA23G1R1	0	0		
2	12	34.718798	56.602989	58.42	67	IIaA15G2R1	1.56	2.51	46.881090	<0.0001
						IIaA16G1R1	0	0		
						IIaA17G2R1	2.48	Infinity		
						IIaA20G1R1	0	0		
						IIaA21G1R1	2.48	Infinity		
						IIaA22G1R1	0.26	0.17		
						IIaA23G1R1	0	0		

RR, relative risk; LLR, log likelihood ratio.

(11, 48, 49), New Zealand (50), Brazil (51), Chile (52), and Colombia (53). However, the results differ from those observed in Sweden (54), China (55, 56), Canada (57), and Ethiopia (58), where *C. bovis* was the most frequently reported species in cattle of this age range.

The analysis of the sequence of the gp60 gene allowed us to identify seven different *C. parvum* subtypes, all within the family IIa, which is widely recognized for its zoonotic potential (59). The subtypes of the IIa family differ from each other in the number of trinucleotides encoding the amino acid serine, as well as in the number of copies of the ACATCA sequence at the end of the sequence. As observed in other studies from South America, including Argentina (11, 48), Brazil (51), Colombia (53), and Chile (60), all detected subtypes in our study also had only one copy of the ACATCA sequence (R1), with variable copy numbers of the TCA trinucleotide (A15, A16, A17, A20, A21, A22, and A23). Unlike studies from neighboring Argentina (11, 48), we identified two subtypes with two copies of the trinucleotide TCG (G), including subtype IIaA15G2R1 that has also been identified in Brazil, Chile, and Colombia (53, 60, 61), as well as subtype IIaA17G2R1 (35, 37, 38) that so far —of the south American countries—had only been identified in Chile (60) and Brazil (62). In this sense, our study broadens the current knowledge on *C. parvum* subtypes infecting cattle in this subcontinent.

Of the seven subtypes identified in our study, five had been found in humans elsewhere (34–40), and are considered zoonotic. At least one of these five zoonotic subtypes was detected in 28/29 (96.6%) of the farms in all departments in our study, indicating a widespread distribution. As described by other studies (13, 14, 43, 44, 63, 64), the most frequent subtype in our study was IIaA15G2R1. In South America, this subtype has been reported in cattle in Brazil (51), Chile (60), and Colombia (53) but not in Argentina (11, 48, 49). The second and third most frequent subtypes identified in our study (IIaA20G1R1 and IIaA22G1R1) are the most frequent ones found in Argentina (11, 48). Subtype IIaA17G2R1 found in our study was reported in cattle in Australia (38, 65), Italy (63), Germany (43), Brazil (62), and Chile (60), as well as in humans in the USA (37), Malaysia (35), and Australia (38). Interestingly, this subtype was involved in an outbreak of diarrhea in a summer camp in the USA, where there was contact between humans and infected calves, with the concurrent identification of this subtype in samples of both species (5). In Brazil, this subtype was found in the feces of calves, as well as in water from dairy farms (62), suggesting water contamination and waterborne transmission. To the best of our knowledge, there is no published information on the identification of *Cryptosporidium* species or subtypes infecting people or any animal species in Uruguay; thus, our study provides novel information that could be used to frame future studies on diagnostics, molecular epidemiology, and risk assessments.

Although we have previously documented that *Cryptosporidium* spp. infection in dairy calves in Uruguay was statistically associated with neonatal diarrhea (23), none of the subtypes found in this study was statistically associated with this clinical manifestation, indicating that diarrhea is associated with *C. parvum* infection regardless of the subtype. The lack of association between some of these *C. parvum* subtypes in neonate calves under similar rearing conditions has also been documented by other authors (11, 48, 53). Additional studies are necessary to determine the eventual implication of different subtypes in terms of their pathogenicity and elucidate the epidemiology of bovine cryptosporidiosis.

Despite the wide geographic distribution and subtype diversity found in our study, two spatial clusters were detected. In the primary cluster, there was a greater risk for farms to have subtypes IIaA20G1R1 and IIaA22G1R1. In the secondary cluster

TABLE 4 | Distance of farms with calves infected with different *Cryptosporidium parvum* subtypes from surface natural watercourses and downstream water treatments plants.

Farm ID	Distance of the calf-rearing areas to the nearest draining surface natural watercourse (m)	Does the watercourse flow downstream into surface water treatment plants?	Surface water treatment plant ID and department	Distance from the watercourse to the downstream water treatment plant (km)	*C. parvum* subtypes and calves	Spatial cluster
1	300	No	–	–	*IIaA15G2R1 (7 calves)	None
2	200	No	–	–	IIaA22G1R1 (2 calves), *IIaA15G2R1 (1 calf)	None
3	20	Yes	San Juan, Colonia	30	*IIaA23G1R1 (6 calves)	None
4	40	No	–	–	*IIaA15G2R1 (3 calves)	Secondary
5	900	No	–	–	IIaA22G1R1 (7 calves), *IIaA15G2R1 (1 calf)	None
6	265	No	–	–	*IIaA15G2R1 (8 calves)	Secondary
7	250	Yes	Bequeló, Soriano	108	IIaA22G1R1 (7 calves)	Primary
8	50	Yes	Santa Lucía (SL) and Aguas Corrientes (AC), Canelones	25.5 (SL), 36.5 (AC)	*IIaA17G2R1 (5 calves)	Secondary
9	700	No	–	–	*IIaA15G2R1 (4 calves)	Secondary
10	600	No	–	–	*IIaA20G1R1 (7 calves)	Primary
11	500	No	–	–	*IIaA20G1R1 (3 calves)	Primary
12	150	Yes	Bequeló, Soriano	101	*IIaA20G1R1 (5 calves)	Primary
13	620	No	–	–	*IIaA20G1R1 (4 calves), *IIaA15G2R1 (1 calf)	Primary
14	300	Yes	Ecilda, San José	20	*IIaA16G1R1 (3 calves)	None
15	90	No	–	–	*IIaA15G2R1 (4 calves)	Secondary
16	365	No	–	–	*IIaA15G2R1 (10 calves)	Secondary
17	340	No	–	–	*IIaA20G1R1 (9 calves), *IIaA15G2R1 (1 calf)	None
18	450	Yes	SL and AC, Canelones	15 (SL), 26 (AC)	*IIaA15G2R1 (5 calves)	Secondary
19	200	No	–	–	*IIaA15G2R1 (5 calves)	None
20	400	No	–	–	*IIaA15G2R1 (3 calves)	None
21	860	No	–	–	IIaA21G1R1 (4 calves), *IIaA15G2R1 (3 calves)	Secondary
22	550	Yes	SL and AC, Canelones	9 (SL), 20 (AC)	*IIaA15G2R1 (1 calf)	Secondary
23	50	No	–	–	*IIaA15G2R1 (6 calves)	Secondary
24	430	No	–	–	*IIaA15G2R1 (5 calves)	Secondary
25	460	Yes	Arroyo Grande, Flores	97	*IIaA15G2R1 (8 calves)	None
26	700	Yes	Arroyo Grande, Flores	94	*IIaA15G2R1 (4 calves), *IIaA20G1R1 (1 calf)	None
27	120	No	–	–	*IIaA20G1R1 (3 calves)	Primary
28	120	No	–	–	*IIaA20G1R1 (8 calves)	Primary
29	180	Yes	SL and AC, Canelones	30 (SL), 41 (AC)	*IIaA15G2R1 (8 calves), IIaA22G1R1 (1 calf)	Secondary

**Subtypes that have been found in humans elsewhere: IIaA15G2R1 (34), IIaA16G1R1 (35, 36), IIaA17G2R1 (35, 37, 38), IIaA20G1R1 (39), and IIaA23G1R1 (40).*

there was increased risk for subtypes IIaA15G2R1, IIaA17G2R1, and IIaA21G1R1. Geographic differences in *Cryptosporidium* species and subtypes have long been described (10, 34, 66). In our study, one of the clusters was located to the north and the other to the south of the Río Negro. This river could have implications as a natural geographic barrier, which would help to explain these differences, also considering that cattle movement across this river is controlled by a sanitary barrier by the Ministry of Livestock, Agriculture, and Fisheries. Additionally, the cluster in the north, adjacent to Argentina, contained the same subtypes that are more frequent in that country. The cluster located in the south presented the most frequent subtype in cases of zoonosis

worldwide (IIaA15G2R1), as well as one involved in waterborne outbreaks of cryptosporidiosis (IIaA17G2R1) (5). As suggested by other authors, the origin of the animals, sources of infection, and management practices probably determine that in some areas there are greater frequencies of certain subtypes (10, 11). Interestingly, one of the spatial clusters identified in our study overlapped with Uruguay's most populated metropolitan area, which includes the capital city, Montevideo. The identification of the geographic distribution and spatial clustering of the subtypes of *C. parvum* facilitate the identification of risk areas for both animals and humans.

It should be considered that cattle movements either within Uruguay or internationally, could eventually determine geographic shifting of different *C. parvum* subtypes over time. In Uruguay, ∼16% of dairy farmers send female dairy calves from the farms where they are born, to be custom raised in distant farms referred to as dairy rearing farms (DRFs) that gather tens of thousands of calves owned by many different farmers (67). Once heifers reach puberty, they are bred in the DRFs and sent back to their farms of origin before calving. In turn, neonate male dairy calves are usually sold and transported to beef farms within the country where they are reared for meat. This indicates that *C. parvum* subtypes detected in spatial clusters in this study could potentially spread to other geographic regions of the country.

Similarly, the international trade of livestock represents a potential way of transboundary dissemination of *C. parvum*, and other pathogens. From 2008 to 2016 Uruguay exported over 1.5 million live cattle head to Turkey (53%), Egypt (15%), China (14%), Brazil (5%), Lebanon (3%), and other destinations (9%) (68). As we will discuss in the following paragraphs, many of the zoonotic *C. parvum* subtypes identified in our study have either a limited occurrence or have not been identified in livestock species in countries importing cattle from Uruguay; thus trading provides a chance for transcontinental spread of these subtypes.

The first study on molecular subtyping of *C. parvum* in Turkey, the main country importing cattle from Uruguay, was published in 2012, and analyzed 13 bovine strains. Subtype IIaA15G2R1, the most frequent subtype found in our study, was identified in 10 animals, while subtype IIaA16G3R1 was identified in 2 animals and subtype IIdA15G1 was found in the remainder (69); these two subtypes were not identified in our study. Later, in 2016, a broader study that identified *C. parvum* in 27 dairy calves and 9 goat kids in Turkey revealed subtypes IIaA13G2R1 (20/23), IIdA18G1 (2/23), and IIdA20G1b (1/23) in cattle, and subtypes IIaA13G2R1 (3/8), IIaA15G1R1 (2/8), IIdA22G1 (2/8), and IIdA18G1 (1/8) in goat kids (70). None of these subtypes were identified in our study. Another Turkish study from 2017, described *C. parvum* in 73 of 112 diarrheic goat kids from 12 goat farms (71). Sequence analysis of the gp60 locus could be achieved in 67 cases, and revealed subtypes IIaA14G1R1 and IIaA15G1R1 in 25 goat kids each, IIdA18G1 (n: 9), and IIdA17G1 (n: 8). None of these subtypes were identified in our study. A more recent and even larger study from this country assessed 415 fecal specimens from diarrheic calves (n: 333), lambs (n: 67), and goat kids (n: 15), and identified *C. parvum* in 90 calves, 13 lambs, and 2 goats kids (72). Of the 11 subtypes detected (IIaA11G2R1, IIaA11G3R1, IIaA12G3R1, IIaA13G2R1, IIaA13G4R1, IIaA14G1R1, IIaA14G3R1, IIaA15G2R1, IIdA16G1, IIdA18G1, IIdA22G1) in 82 cases (70 calves, 10 lambs and 2 goat kids), only one (IIaA15G2R1, identified in 4 calves and 3 lambs) was found in our study. Another recent study from Turkey identified *C. parvum* in 138 of 550 calves and heifers (73). Gp60 gene sequence analysis reveled only two subtypes (IIaA13G2R1, IIaA14G1R1) in all 138 samples, none of which were identified in our study. Altogether, this indicates that IIaA15G2R1, the most common subtype identified in Uruguay and regarded as an hyper-transmissible zoonotic subtype, is uncommon in livestock in Turkey (so far only identified in 14 cattle and 3 lambs), and that all other subtypes found in cattle in Uruguay have not been detected in livestock in Turkey, so livestock trading could represents a risk of introduction of these subtypes in the country.

A study on molecular epidemiology of *Cryptosporidium* spp. in 804 livestock (cattle and buffalo) and 165 humans in Egypt sampled in April-June 2011 (74), revealed an overall prevalence of *Cryptosporidium* spp. of 32.3% in livestock (260 animals), and 49.1% in humans (81 cases). *C. parvum* was identified in 142 livestock and 32 humans. All *C. parvum*-positive samples for which a nested gp60 PCR product was obtained were sequenced (n: 120); subtype family IId (which was not identified in our study) was significantly more frequent than subtype family IIa. All *C. parvum* of subtype family IIa detected from cattle (22.5%) and buffalo (11.4%) belonged to the IIaA15G1R1 subtype (which was not identified in our study), while human infections with subtype family IIa (50%) were found to be caused by subtypes IIaA15G1R1 (n: 2, subtype not identified in our study) and IIaA15G2R1 (n: 5, subtype most frequently identified in our study). Another study from Egypt evaluated the prevalence and molecular characteristics of *Cryptosporidium* spp. in dairy cattle in four Nile River delta provinces (75). *Cryptosporidium* spp. were identified in 13.6% of 1,974 fecal specimens obtained from 12 farms between December 2009 and November 2011. Successful amplification and sequencing of the gp60 locus for *C. parvum* subtyping was possible in 37 specimens, 27 were identified as IIaA15G1R1, 9 as IIdA20G1, and 1 as IIaA14G1R1r1b. None of these subtypes were identified in Uruguay. Another study on prevalence and genotyping of *Cryptosporidium* spp. in farm animals in Egypt that evaluated 466 samples from buffalo, 1,697 from cattle and 120 from sheep, identified *C. parvum* in 2 buffalo, 23 cattle and 0 sheep, the subtypes involved being IIdA20G1 and IIaA15G1R1 (76), none of which were identified in our study. Lastly, another study on *Cryptosporidium* genotypes and subtypes in dairy calves in Egypt identified *C. parvum* in 24 from 96 sampled calves, 23 were subtyped as IIdA20G1 (not identified in our study), and only 1 as IIaA15G2R1 (77), which was the most frequent subtype identified in our study. In summary, the most frequent subtype found in Uruguay (IIaA15G2R1), which has been regarded as an hyper-transmissible subtype, has a limited occurrence in Egypt, and to the best of our knowledge, it has so far only been identified in one dairy calf (77) and 5 human patients (74) in this country. In this context, livestock exports from Uruguay could represent a risk of introduction of *C. parvum* subtypes that are either infrequently

identified or have not been identified in livestock and people in Egypt.

In China, there is a high diversity of *Cryptosporidium* spp. and subtypes, and the dominant *C. parvum* subtypes detected in this country are rarely detected in other countries. Domestic ruminants (calves, lambs, goat kids) are mostly infected with non-pathogenic *Cryptosporidium* spp., such as *C. bovis* (calves) or *C. xiaoi* (lambs and goat kids). *C. parvum* started to appear in dairy calves as a consequence of concentrated animal feeding operations. Subtyping of *C. parvum* in 9 studies involving dairy calves in 8 geographic areas of China published between 2011 and 2017 identified the exclusive occurrence of IId subtypes, mostly IIdA15G1 and IdA19G1 (78). The few IIa subtypes identified in cattle in China include IIaA15G2R1 (n: 8), IIaA16G2R1 (n: 2), IIaA14G1R1 (n: 1), IIaA14G2R1 (n: 1), and IIaA16G3R1 (n: 1), which were geographically restricted to the Qinghai province (79). Of these subtypes rarely detected in Chinese cattle, only the hyper-transmissible subtype IIaA15G2R1 was identified in Uruguay. *C. parvum* subtypes have also been identified in other domestic ruminants in China, including yak, sheep, and goats. One study identified the exclusive occurrence of a few IIa subtypes in yak, including IIaA15G2R1 (n: 8), IIaA16G2R1 (n: 2), IIaA14G1R1 (n: 1), IIaA14G2R1 (n: 1) and IIaA16G3R1 (n: 1) (80). The IIa subtypes identified in sheep include IIaA15G2R1 and IIaA17G2R1 (81), and those identified in goats include IIaA14G2R1, IIaA15G1R1, IIaA15G2R1 and IIaA17G2R1 (82). Of these seven IIa subtypes identified in yak, sheep, and goats in China, only subtypes IIaA15G2R1 and IIaA17G2R1 were identified in calves in Uruguay. In short, cattle imports from Uruguay could potentially determine the introduction of IIa subtypes that have not been detected or have a limited occurrence in livestock in most Chinese provinces.

Because *Cryptosporidium* is one of the most important waterborne parasites worldwide (7), and given the risk for contamination of aquatic environments by infected animals (57, 83), we assessed the distance between the calf-rearing areas in the studied farms and the closest natural surface watercourses. This was performed by considering the shortest natural drainage route of the farmlands, as well as whether these watercourses would flow downstream into public water treatment plants harvesting surface water for human consumption. This approach revealed some interesting observations. For instance, we found that watercourses draining four farms in the secondary spatial cluster flowed downstream into the Santa Lucía river and further down into Aguas Corrientes water treatment plant, which also overlapped with the secondary spatial cluster. Aguas Corrientes is the country's main water treatment plant, supplying drinking water to ∼1.7 million people in the largest metropolitan area in the departments of Montevideo and Canelones (84). The calf-rearing areas in these four farms, lodging 19 calves infected with the zoonotic subtypes IIaA17G2R1 and IIaA15G2R1, were 50, 180, 450, and 550 m away from their respective closest surface watercourses. The calf-rearing area that was closest (20 m) to a natural watercourse was in farm three and lodged six calves infected with the zoonotic subtype IIaA23G1R1. This watercourse flows down into the San Juan water treatment plant in the department of Colonia. Additionally, it should be stated that all the watercourses draining farmlands in this study eventually flow downstream into the Río Negro, Río Uruguay and/or Río de La Plata, all of which line the coast of recreational freshwater beaches in the departments of Río Negro, Soriano, Colonia, San José, Montevideo, and Canelones.

Studies that assessed the presence and concentration of *Cryptosporidium* oocysts in surface watercourses located upstream and downstream from cattle farms found higher proportions and concentrations of oocysts in downstream samples, suggesting that cattle represent a source of surface water contamination with this parasite (57, 85). In one of these studies, the natural watercourses were located within 500 m of the cattle housing facilities (57). Based on this information, it is reasonable to speculate that water draining from the calf-rearing areas in our study (particularly those housing calves outdoors on dirt floor where feces were not removed from the calf-rearing areas) could eventually act as a vehicle of *C. parvum* oocysts to the respective natural watercourses, i.e., after heavy rainfalls leading to surface runoff or floods. Taken together, our results indicate that water contamination by oocysts of zoonotic subtypes of *C. parvum* shed by cattle in Uruguay is likely and could represent a potential risk to public health if people are exposed to natural watercourses or if water used for drinking, recreation, or crop/produce irrigation is not sanitized properly.

Flooding events are frequent in Uruguay. In 2015 and 2016 the El Niño phenomenon caused extremely unstable climatic conditions in the region, causing rivers to swell and overflow their banks. In 2016, the same year of the sampling in our study, the accumulated rainfall in the country was 1,268 mm, 207 mm (19.5%) higher than the average annual accumulated rainfall in the previous decade (2006–2015), which was of 1,061 mm (86). In April of 2016, heavy rains and a tornado caused floods and thousands of flood victims in the entire country, according to Uruguay's national emergency system (87). Such extreme climatic conditions facilitate the environmental contamination with waterborne disease agents.

Additionally, surface water in Uruguay is used by farmers to irrigate crops and produce, which could also represent a possible source of transmission of *C. parvum* to humans through contaminated soil and vegetables (88). Another potential way of exposure of humans to untreated surface freshwater is through recreation. Activities such as swimming, sailing, kayaking, canoeing, fishing, waterskiing, windsurfing, and kiteboarding are commonly practiced in Uruguay by inhabitants and tourists alike, which in 2016 Uruguay accounted for over 3.3 million tourists (89). Furthermore, agritourism is expanding in the country, and some farms offer hands-on experiences with livestock, such as milking cows or herding cattle (90). It should be noted that human outbreaks of cryptosporidiosis with proven bovine-to-human *C. parvum* transmission have been documented in recreational spring pasture events in Sweden (8).

Water contamination probably perpetuates the transmission cycle of cryptosporidiosis in cattle in Uruguay. In all farms included in this study, calves were administered untreated underground water (**Supplementary Table 1**). In a previous

study we found group A rotavirus (RVA) RNA as well as viable and infective RVA particles in drinking water sources administered to neonate dairy calves in Uruguayan farms (91). This indicates that water likely acts as a vehicle in the transmission of causative agents of neonatal calf diarrhea that are less resistant in the environment than *Cryptosporidium* spp. oocysts. Unfortunately, we have not been able to validate laboratory tests to efficiently identify *Cryptosporidium* spp. in water samples.

In Uruguay, 90% of treated water is obtained from superficial sources and undergoes conventional treatment, a physicochemical process consisting of 6 phases: A- pre-treatment, B- coagulation with aluminum sulfate, C- flocculation, D- sedimentation (or flotation), E- filtration, and F- disinfection (chlorination) (92). *Cryptosporidium* spp. oocysts can be physically removed from water supplies by conventional particle separation processes including chemical coagulation-flocculation, sedimentation, and granular media filtration (93). Although conventional water treatment can be effective in reducing *Cryptosporidium* spp. oocyst loads, the effectiveness depends on the initial oocyst concentration in the source water. Additional special physical and chemical processes such as pressure-driven membrane microfiltration or ultrafiltration, or special disinfection procedures such as treatment with ozone or ultraviolet light irradiation, may be required to inactivate *Cryptosporidium* spp. oocysts, as the parasite is highly resistant to chlorination (even at very high doses after prolonged contact time) (93). To the best of our knowledge, such procedures are not available in Uruguayan water treatment plants and drinking water in the country is not specifically screened for *Cryptosporidium* spp. before, during or after the potabilization process. Major waterborne outbreaks of cryptosporidiosis have been linked to evidence of suboptimal water treatment in other countries (6).

CONCLUSION

C. parvum infection is widespread in dairy calves in Uruguay, and calves are reservoirs of zoonotic *C. parvum* subtypes, the most frequent being IIaA15G2R1 and IIaA20G1R1. Spatial clustering of zoonotic *C. parvum* subtypes in cattle overlapping with highly populated metropolitan areas and natural surface watercourses that flow downstream into public water treatment plants, including the country's main water plant harvesting surface water for human consumption, raises a concern for potential zoonotic waterborne transmission.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal sampling protocol was approved by INIA's animal ethics committee for the use of animals in experimentation (CEUA, protocol # 20199). Verbal informed consent for participation was obtained from the owners of the calves/farms.

AUTHOR CONTRIBUTIONS

FG, FR-C, and RC conceived the study. RC, MM, and BS performed molecular testing and DNA sequence analyses. RC and LC-L built the maps and analyzed data. CP-R analyzed data. RC and FG wrote the first draft and final version of the manuscript. MM, LC-L, CP-R, and FR-C wrote parts and/or edited the manuscript. All authors read and approved the content of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00562/full#supplementary-material

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Corrigendum: Dairy Calves in Uruguay Are Reservoirs of Zoonotic Subtypes of *Cryptosporidium parvum* and Pose a Potential Risk of Surface Water Contamination

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In the original article, there was an error in the acknowledgments section. The names of three persons and a staff group were omitted.

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New Insights Into the Peculiar World of the Shepherd-Dog Parasites: An Overview From Maremma (Tuscany, Italy)

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Several developments have been recently achieved to understand pet-dog parasites and their relationship with hosts; however, parasites' presence and distribution in shepherd-dog have been mainly neglected; this knowledge gap is of critical sanitary importance, as shepherd-dogs could harbor zoonotic helminths including *Echinococcus granulosus sensu lato.* The related human disease, cystic echinococcosis, is a worldwide neglected disease, with high endemicity in the Mediterranean Basin. To evaluate the presence of *E. granulosus* and other parasites, a sheep-dog population from the province of Grosseto (Tuscany, Italy) has been investigated. Overall, 648 dog fecal samples obtained from 50 modern sheep farms, having a total of 216 dogs, were collected. Specimens were analyzed using a standardized centrifugal flotation method (specific gravity = 1.3). Taeniid eggs detected were further isolated using a sieving/flotation technique. DNA was isolated from eggs for PCR and sequence analyses for species identification (gene target: 12S rRNA and *nad1*). Thirty-nine (78%) farms tested positive for at least one parasite species or genus. The most represented intestinal helminths were *Toxocara* spp. in 64% of farms, followed by Ancylostomatidae (58%), *Trichuris vulpis* (50%), *Capillaria* spp. (34%), and taeniids (32%). Sequence analyses confirmed the presence of *Taenia hydatigena* in seven farms, *Taenia* (syn. *Multiceps*) *multiceps* in five farms, and *T. pisiformis* in one farm. No DNA was extracted from four previously taeniid egg-positive farms. No amplification of amplicon corresponding to *E. granulosus* was achieved in the investigated farms. Although not entirely expected, Spearman's test showed a positive correlation between flock size and the number of dogs per farm ($\rho = 0.588$, $P < 0.001$). The quantitative analysis reported that the home slaughter practice was affected neither by the flock size nor by the number of dogs per farm. The probability to diagnose farms positive for taeniids had been increased by about 35% for each dog unit increase [odds ratio (OR) = 1.35, $P = 0.012$]. In conclusion, the wide distribution of *T. hydatigena* and *T. multiceps* detected in the present study clearly reveals that dogs have still access to raw offal, a major risk for the transmission of *E. granulosus*. Home slaughtering is an unavoidable practice, and more efforts must be undertaken by the public health system to prevent and control potential zoonotic taeniids.

Keywords: shepherd-dog, parasites, taeniids, *E. granulosus*, epidemiology, public health

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INTRODUCTION

Although progress has been recently made to increase scientific knowledge of pet-dog parasites (1), the same has not been done for shepherd-dog helminths. The incommunicability between pastoralist world and public health system makes the picture even hazier. This is primarily due to the different interests involving the two categories (2). This knowledge gap is of critical sanitary importance, as shepherd-dogs could harbor potentially zoonotic parasites, such as *Echinococcus granulosus sensu lato*, *Taenia* spp., *Taenia* (syn. *Multiceps*) *multiceps*, and *Taenia serialis*, whose life cycles include the dog as the definitive host and the sheep or other herbivorous as the intermediate host (3, 4). Additionally, cystic echinococcosis (CE), caused by intermediate larval stages of *E. granulosus*, is among the five most frequently diagnosed zoonosis in the Mediterranean Basin (5) and distributed worldwide (6). CE appears differently distributed across the Italian peninsula, showing a hyper-endemic diffusion in the south and being considered sporadic in the north (7, 8). This should not be surprising since sheep domestication started around the fifth century B.C. in the Fertile Crescent (9), and dog breeding for guard and hunting intents started around 15,000 B.P. (10). Since then, dogs and sheep have maintained a strong connection at farm level, sharing parasites. In this context, shepherds play a crucial role in the spread of metacestodoses between sheep and dog by feeding dogs with raw sheep meat and offal, which have been directly slaughtered and butchered on the farm. For example, Singh et al. (11) report that around 60% of the interviewed farmers from New Zealand fed dogs by using home-slaughtered "meat."

Diagnosis and detection of *E. granulosus* into the definitive host are key points in developing epidemiological studies and implementing hydatid control programs in endemic areas (12). Generally, two paths are available to detect taeniids from the small intestine: *ante-mortem* and *post-mortem* examinations. Obviously, the latter is not always possible, although necropsy has shown 100% specificity and 97% sensitivity, even at a very low parasite burdens (<6 worms) (12) and remains the gold standard for the detection of adult tapeworms (13). However, both may impose a risk to public health; therefore, appropriate measures must be taken to reduce the zoonotic impact (13). *In-vitam* examination is performed through several laboratory techniques using different matrices, such as sera and feces. Indirect diagnosis, such as ELISA tests performed on serum, have been attempted showing variable sensitivities, ranging from 40 to 90% (14); however, they are not routinely used. Tests for the detection of *Echinococcus* coproantigens based on ELISAs have been developed by several research groups (12, 15). These tests have been used mainly in control programs, although some cross-reactions with other intestinal cestodes have been observed (16). On the other hand, molecular analysis of feces showed very low sensitivity, as it yields 74% false-negative results when performed from 21 to 31 days post-infection (17). Furthermore, copro-PCR is challenging even after 31 days post-infection, as DNA extraction from fecal samples is complicated by the presence of inhibitory substances (12).

All the tests mentioned above share the feature of being useful for monospecific parasite detection (18). Parasite concentration by coprological flotation is a classical approach for a variety of intestinal parasites, with variable specificity and sensitivity restricted to the patent period only (19). Parasite stages excreted with feces can be classically differentiated by the morphology of eggs, cysts, or oocysts and more precisely by morphometry (e.g., *Toxocara* spp., hookworms, *Capillaria* spp., and oocysts) (20).

Taeniid eggs, which are discontinuously shed, cannot be differentiated by light microscopy (21).

The detection of the eggs in fecal samples after concentration by traditional routine diagnostic methods is claimed to suffer from low sensitivity (21); however, so far, it has not been evaluated for all taeniid species. The enrichment of taeniid eggs and their subsequent genetic analysis can overcome this limitation and open new diagnostic strategies. Efficient enrichment of taeniid eggs was achieved by a combination of sequential sieving and flotation in zinc chloride solution (F/Si method) (22). In a field study in Lithuania, significantly more dogs excreting taeniid eggs were diagnosed by the F/Si method (34 of 240 dogs investigated) as compared with 12 positive animals identified with the modified McMaster method, an approach known to have low sensitivity. Genetic analyses performed on the 34 egg sediments identified by the F/Si method revealed nine *E. granulosus s.l.* and two *Echinococcus multilocularis* infections, but only one of these *Echinococcus*-positive animals was identified when using the McMaster method as a screening test (23), documenting, that in general lower eggs per gram (EPGs) are present in *Echinococcus* as compared with *Taenia* infections. However, other screening methods for the isolation of taeniid eggs were more sensitive and comparable with the F/Si method (i.e., flotation Ovassay technique) (24–26).

Following egg isolation with any of the aforementioned methods, genetic analyses with specific primers can be performed [primers for *Echinococcus* spp.; see (12, 18)]. A poly-specific approach based on targets in mitochondrial genes with a multiplex PCR allows the differentiation among *E. multilocularis*, *E. granulosus s.l.* other cestodes from canines (27). Sequence analyses of the amplicons for "other cestodes" allow further identification of some *Taenia* spp. (*Taenia hydatigena*, *Taenia ovis*, *Taenia taeniaeformis*, *Taenia polyacantha*, *Taenia pisiformis*, and *Taenia crassiceps* but cannot clearly differentiate between *T. multiceps* and *Taenia krabbei* with the currently available molecular data). Identification of *Taenia* to the species level is of value in *Echinococcus* control programs or in very low endemic areas to trace back *Taenia* infections in dogs, typically originating after ingestion of infected farm animals (*T. hydatigena*, *T. ovis*, and *T. multiceps*) or from rodent and lagomorph intermediate hosts (*T. crassiceps*, *T. polyacantha*, *T. taeniaeformis*, and *T. serialis*) (18).

Furthermore, this poly-specific approach has successfully been used in investigations of wild carnivores, in foxes (18), or wolves in Italy (28) and Portugal (29) documenting their involvement in taeniid cycles.

Due to the lack of available data, this field study aims to contribute to the knowledge about the frequency of shepherd-dog

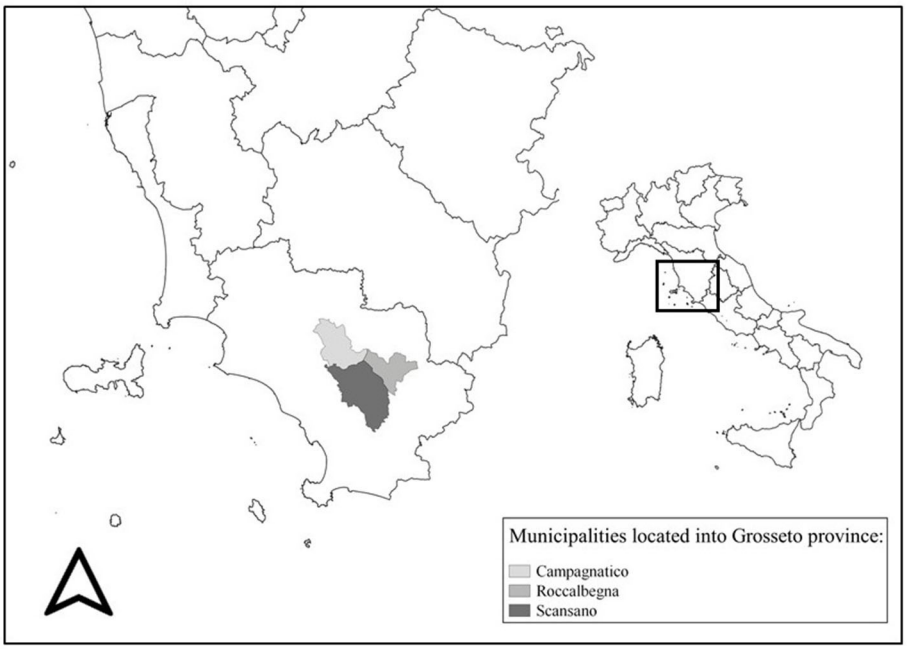

FIGURE 1 | Gray scale represents the three municipalities where the involved farms were located.

parasites at farm level, mainly focusing on *E. granulosus* in an endemic area in Tuscany region, Central Italy (7).

MATERIALS AND METHODS

Study Area

The activity was performed in the Southern area of Tuscany (province of Grosseto, Central Italy), named Maremma (from Latin *maritima*, "maritime") (**Figure 1**). This subregion is suited to animal breeding, namely, sheep, cattle, and horses. The area extends for about 5,000 km^2, covering two regions and five provinces. The hilly municipalities of Campagnatico, Roccalbegna, and Scansano, within the Grosseto province, consist of an area of nearly 56,000 ha, where a total of 46,238 sheep heads over 203 breeding units are farmed. The 203 registered farms in the area are distributed as follows: 51 with 14,920 heads in Campagnatico's municipality; 38 in Roccalbegna hosting 7,784 heads, and 114 in Scansano with 23,534 heads.

Inclusion Criteria and Fecal Sampling

A preliminary list of the total number of the farms was provided by Public Health Services veterinarians and workers of CIA (Confederazione Italiana Agricoltori). Firstly, farms not having dogs were excluded from the study. Furthermore, only farms with at least one dog, able to either have contact with the sheep flock or accede to the pastures, were included. Finally, also based on the willingness of the farmers to be involved, a subset of 50 farms remained in the study.

The selected farms were visited between May 2016 and February 2017. Animal-level and farm-level data were collected during each visit. Farmers were asked to answer a questionnaire, and data were entered into MS Excel (Microsoft Inc., Sacramento, California, USA). Animal-level information included the number and the species of farmed animals and the number of dogs in the farms. Farm-level information included the sheep production system (meat, dairy, or both), prophylactic measures against infectious diseases, and GPS coordinates. Data reporting home slaughter practices were also obtained during the visit.

Randomly walking throughout the property at the time of the visit, three dog fecal samples, detected directly on the ground, were picked up per dog present on the farm. Each fecal sample was labeled and stored into a plastic bag individually and subsequently placed into a refrigerated container/bag. As a biosafety precaution, samples were stored for 10 days at −80°C (30) and then at −20°C until examination.

Parasite Collection

Up to 5 g of feces for each sample was analyzed. Parasite elements were concentrated from fecal specimens by using the Di Felice and Ferretti (31) solution (sodium nitrate and sugar; specific gravity = 1.3) as flotation media in a standardized centrifugal flotation method (32). When positive for taeniid eggs, the corresponding leftover samples were stored at −20°C for further egg isolation/PCR aimed at achieving species identification.

Genetic Identification of Taeniid Eggs

Taeniid eggs were isolated with a combination of flotation in zinc chloride solution followed by sequential sieving (F/Si method) (22). DNA extraction was carried out following Štefanić et al. (33), and species identification of taeniid egg was performed using a multiplex PCR, according to Trachsel et al. (27), using a Qiagen multiplex PCR kit (Qiagen,

Hilden, Germany). Furthermore, "other cestode" amplicons were sequenced, after purification using the MinElute PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing was performed by Microsynth, Switzerland. Sequencing results were compared with reference mitochondrial genes for all taeniid species retrieved from GenBank.

Statistical Analysis

Data collected through in-person questionnaires were merged with laboratory results into a MS Excel spreadsheet and then imported to Stata 15 (StataCorp LLC, College Station, Texas, USA) for analyses. The number of sheep and dogs was interpreted as independent variables and correlated to the home slaughter practice and the presence/absence of taeniids into the farms. Continuous data that were non-normally distributed were summarized using medians and inter-quartile ranges (IQRs), while normally distributed data were summarized using mean ± *SD*. Spearman's correlation test was computed to assess the relationship between sheep and dogs, as their association is not always, at least in Italy, rational and predictable. Fisher's exact test was applied, as more than 20% of cells had expected frequencies < 5, to compare groups (34). When reasonable, odds ratios (OR) and relative 95% confidence intervals (CIs) have been assessed as measures of association to explore the effect of independent over dependent variables. Results were considered significant when $P \leq 0.05$.

TABLE 1 | Number of farms with the specific number of dogs, their frequency, and the relative collected samples.

No. of dogs *per* farm	No. of farms	Freq %	No. of collected samples
1	4	8	3
2	12	24	6
3	9	18	9
4	9	18	12
5	1	2	15
6	3	6	18
7	3	6	21
8	4	8	24
9	2	4	27
10	2	4	30
11	1	2	33

RESULTS

A total of 50 sheep farms were visited from May 2016 to February 2017. Overall, farms hosted 20,388 sheep with a median of 347.5 ranging from 15 to 2,095 heads (IQR: 173–460). Dogs, including livestock guarding dogs, shepherd-dogs, hunting dogs, and pets, accounted for a total of 216 (**Table 1** shows dogs' frequency by farm), showing a mean of 4.3 ± 2.72 with a range of 1–11. Considering the livestock guarding dogs and shepherd-dog categories, the average dog/sheep ratio was 1:114.5. Farms with one dog had a mean of 79.75 (*SD* ± 42.1) sheep, while the only farm with 11 dogs had 783 heads. Spearman's correlation test highlighted that the number of dogs hosted in the farms

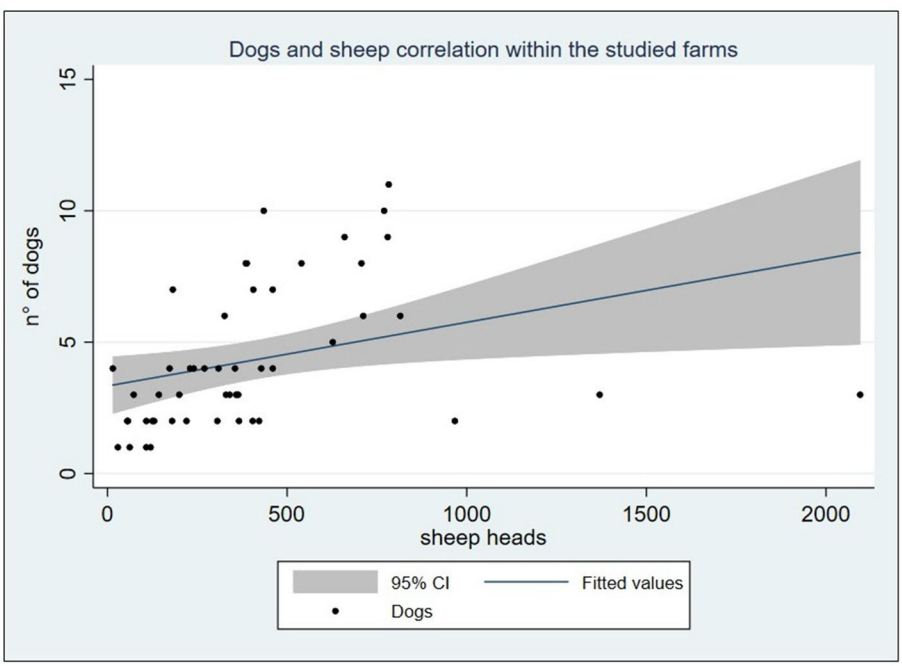

FIGURE 2 | Graph reporting the positive linear correlation between the sheep and dog count within the studied farms ($\rho = 0.588$, $P < 0.0001$).

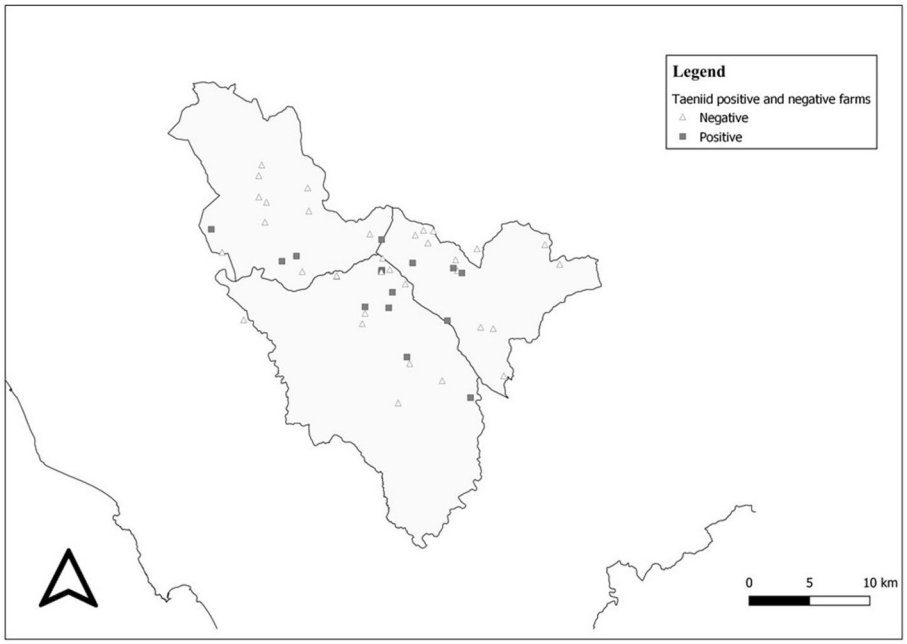

FIGURE 3 | Taeniid-positive and taeniid-negative farms distributed within the study area.

was positively correlated to the number of sheep ($\rho = 0.588$, $P < 0.0001$), as shown in **Figure 2**. Farms included in the study were mostly dairy farms (70%), while 10 were specialized in the production of sheep meat, and only three had both. Home slaughter appeared to be widely spread among farms, since 40 out of 50 farmers declared to practice it. No statistically significant differences emerged comparing the different production systems and the home slaughter practice (Fisher's exact $\chi^2 = 3.581$, $P = 0.26$).

Concerning dog prophylaxis procedures, only 19 farmers out of 50 (38%) reported the administration of anthelmintic drugs to their dogs and stated that they either did not remember the used products or had administered off-label avermectins. None of the farmers mentioned the use of praziquantel, which is the appropriate treatment for taeniids.

A total of 648 fecal samples, based on 216 dogs present into the 50 visited farms, were processed and investigated for the presence of parasites. The crude coprological results revealed a high proportion of positivity for parasites at the farm level since 78% (39/50) were positive for at least one parasite (**Figure 3**). Out of 648 samples analyzed, 312 showed parasitic elements resulting in a 48.1% frequency. **Table 2** shows the five groups of parasites diagnosed by using flotation technique. Specimens with multiple infections were slightly less common than the single infection, with 54.8 and 45.2%, respectively. On the contrary, farms having multiple parasite species were far more common than those with single-parasite type, with 89.7 and 10.3%, respectively (**Table 3**).

A total of 47 fecal samples from 16 different farms (farm-level frequency: 32%) revealed taeniid eggs. PCR product after multiplex PCR for taeniids was acquired in 34 out of 47 positive samples. In terms of frequency among positive samples for taeniids, *Taenia hydatigena* was the most common isolated species (31.9%), followed by *Taenia* (syn. *Multiceps*) *multiceps* (21.3%) and *Taenia pisiformis* (2.1%). Electropherograms from eight sequences were not of high quality or were too short to be able to identify the species; then they were identified as *Taenia* spp. No sample was positive for *Echinococcus multilocularis*

TABLE 2 | Reported frequencies of detected parasites in the surveyed farms.

Recovered parasites at farm level	No. of positive farms	Frequency%
Toxocara spp.	32	64
Ancylostomatidae	29	58
Trichuris vulpis	25	50
Capillaria spp.	17	34
Taeniids	16	32

TABLE 3 | Proportions of single and multiple parasite species detected in the 39 positive farms and in the 312 fecal positive-diagnosed samples.

No. of different parasitism	Farm level		Fecal samples	
	n	%	*n*	%
1	4	10.3	171	54.8
2	8	20.5	100	32.1
3	14	35.9	34	10.9
4	8	20.5	6	1.9
5	5	12.8	1	0.3
Total	39	100	312	100

TABLE 4 | Farm-level odds ratios, *P*-values, and relative 95% CIs among farmer-reported predictors and outcomes.

Outcomes	Predictors	Odds ratios	*P*-values	95% CIs
Home slaughtering	Flock size	1.001	0.382	0.99–1.003
Home slaughtering	No. of dogs	1.12	0.421	0.84–1.49
Taeniids	No. of dogs	1.35	0.012*	1.07–1.72

Statistically significant result.

and/or *E. granulosus s.l.* As regards taeniids' frequency at farm level, *T. hydatigena* was the most commonly detected species at 43.7% (seven out of 16 taeniid positive farms), followed by *T. multiceps* 31.2%; and one farm was positive for *T. pisiformis*. No PCR product was obtained in samples from four different farms.

Three farms were detected having multiple taeniids infections, particularly one with *T. hydatigena*, *T. multiceps*, and *Taenia* spp.; one with *T. multiceps* and *Taenia* spp.; and finally one with *T. multiceps* and *Taenia* spp. No taeniid eggs were detected in home slaughter-free farms (10 out of 50). Twenty-seven farmers reported to have knowledge of CE; eight of them stated to have seen at least one hydatid cyst.

Quantitative analysis, carried out by estimating the ORs, suggested that home slaughter practice was not affected by the flock size. Similarly, the number of dogs did not increase the probability to perform a home slaughtering. On the other hand, for each dog unit increase into the farm, the chance of having circulating taeniids increased by a factor of around 35% (OR = 1.35, $P = 0.012$). Quantitative results are summarized in **Table 4**. Finally, differences in circulating taeniids among different production systems did not show any statistical significance (Fisher's exact = 2.32, $P = 0.32$).

DISCUSSION

The present study offered useful information on the distribution of endoparasites, particularly taeniids, in shepherd-dogs from Maremma's (Tuscany region) sheep farms and provided interesting insights on a few practices commonly adopted by farmers.

Farmers participating with the study were breeding 20,388 sheep, representing almost half of the total number of sheep in the three municipalities; furthermore, the 50 farms considered for the study represented around one fourth of the total 203 sheep farms registered in the area. Assuming that the larger the flock size the more advanced the management systems adopted by the farm, the sampled farms were slightly more technologized than the non-responding ones; this aspect might have represented a selection bias, which resulted in an underestimation of parasite frequencies.

Data on the optimal dogs/sheep ratio providing the best benefit in terms of livestock guarding or herding are lacking. On average, the dog/sheep ratio of 1:114.5 was reasonable as recommended by Gemmell et al. (35), who also urged a drastic reduction and control of dog population size, as a pillar in the control of echinococcosis. As regards the studied farms, Spearman's correlation showed a moderate positive association between the number of dogs per farm and the flock size, demonstrating a non-random allocation. Knowledge about the existence of a rational association among flock size and dog units is useful, if not essential, for the control of zoonoses related to shepherd-dogs (35). All the dogs present into the farms were officially registered, and no stray dogs were reported in the study area.

Farmers did not report which drugs were used as a treatment or as prophylaxes for canine parasitic diseases, which is a commonly noticed behavior (36, 37). Farmers tend to treat dogs using products for sheep simultaneously when deworming the flocks; these products are mostly represented by avermectins, which are useless for taeniids. Furthermore, our results highlighted the presence of nematodes and cestodes, pointing out how this approach is both futile and antieconomic. This practice confirms that shepherd-dog parasites are widely neglected and underestimated among breeders. Attention to dog health issues is insufficient, probably because dogs are not considered as a direct source of income, contrary to the sheep flock.

Diagnosed nematodes are commonly reported also from pets (i.e., ascarids, hookworms, and trichurids) (1). Ascarids were the most common nematodes detected on the farms. The different modes of infection transmission and resistance of the eggs in the environment may lead to a cumulative environmental contamination, representing a risk for human infection (38). Hookworms were found in 29 farms; this result is consistent with the prevailing opinion that these parasites are related to rural environments (39). Additionally, the zoonotic potential of hookworms should not be underestimated as they may induce two severe conditions known as human gut disease (eosinophilic enteritis) and cutaneous larva migrans (CLM) or creeping eruption (40). As for *Trichuris vulpis*, its zoonotic potential is still being debated. Cases of visceral larva migrans (VLM), described in the literature, have been reviewed by Traversa (41), even though dog whipworms are generally not reported as zoonotic pet intestinal nematodes (42).

By comparing our results with data from studies regarding feces randomly collected from soil in urbanized areas of Italy that report positivity of always around 17%, we recorded a higher frequency per sample (43, 44). Additionally, a conference abstract of a nationwide study on owned dogs with constant or regular access to the outdoors, carried out in Italy by Brianti et al. (45), reports a much higher overall prevalence of around 30%. When our frequencies are compared with results obtained within a similar environment, percentages are close (46). Nevertheless, an underestimation of the real prevalence is likely in this study. This may be due primarily to the low sensitivity of the flotation technique (47) and secondly to the effect of freezing, which has been reported to mask low-intensity infections (48). As for farm level, parasites were recovered in approximately 80% surveyed farms, with 90% of positive farms showing multiple parasite infections. These data are higher than the data of Phythian et al. (49), who reported 50% positive farms in a survey carried out in South-Western England. This difference might be due to different sampling methods: in the present study, three stools from each

dog present in the farm were randomly collected on the ground (see **Table 1**), whereas Phytian et al. (49) only sampled one stool. Particularly noteworthy is the fact that, as expected, multiple infections were more common at the farm level than at the sample level, where the majority of samples resulted positive for a single-parasite group (**Table 3**). This could be due to the fact that a farm may have more than one dog mono-parasitized by different parasites.

As often reported, taeniid presence within farms is strictly related to the traditional home slaughtering: indeed, taeniid eggs were not detected in the 10 farms where home slaughter was not reported. Adult sheep meat trade is currently increasing due to the spread of *halal* food market and to typical recipes of the Italian gastronomy, such as sheep skewers, salami, and ham.

Our results highlight how home slaughtering without any veterinary control remains the major risk for the spreading of potentially zoonotic tapeworms. *Taenia hydatigena* was the most represented tapeworm, with around 44% of the farms being positives. A quite recent systematic review focused on the zoonotic potential linked to parasites of carnivores in Iran (50), encompassing studies from 1997 to 2015, reported that *T. hydatigena* was the most frequently isolated parasite in dogs, with a prevalence of around 30% out of 1,539 examined dogs. Usually, *T. hydatigena* represents the most common taeniid species detected worldwide in both domestic (51) and wild environment (28), where the wolf act as the main definitive host (52). This ecological success is probably due to the short period of 5–8 weeks required for the maturation of cysticerci (53).

The second most common tapeworm species identified in this study was *Taenia multiceps*, whose larval stage is a coenurus mainly localized in the central nervous system of small ruminants, which produces a well-known clinical syndrome. *T. multiceps* is worldwide distributed, mainly reported in young animals between 3 and 6 months of age and, accidentally, in adult sheep younger than 18 months (54). Many mammals, including sheep, goats, horses, cattle, camels, deer, and pigs, may serve as intermediate hosts (55). The associated disease in sheep is named "gid" or "sturdy" and has an acute or chronic phase. Due to the shorter prepatent period compared to *E. granulosus*, both clinical presentations might be a warning against the habit to give raw offal to dogs. Additionally, *T. multiceps* coenurosis is a zoonotic infection with more than 50 human cases described in the literature (56), several of which have also been reported in Italy (57), including five from Sardinia (58).

The single sample positive to *Taenia pisiformis* in a farm, where domestic rabbits were absent, suggests the administration of hunting offal.

As expected, none of the samples were positive for *Echinococcus multilocularis*, which has never been reported from the area; surprisingly, *E. granulosus* (sheep-strain G1) was not detected either. Sardinia, where a hyper-endemic scenario for *E. granulosus* is present, reports the highest frequencies around 10%, depending on which ELISA test was utilized (36). Since *E. granulosus* is considered diffused all over Italy (8), its absence in the present study could be linked to the positive feedback of a specific educational course offered by Public Veterinary Services to farmers a few years earlier (as reported by farmers during the visits). However, the wide presence of *T. hydatigena* and *T. multiceps* clearly shows that dogs have still access to raw offal, a major risk for the transmission of *E. granulosus*. These data might also suggest that farmers have learned to recognize and discard only hydatid cysts. Unfortunately, no target DNA was amplified from 14 previously positive samples, possibly due to the low burden of infection. Likewise, in eight samples, it was not possible to identify the *Taenia* species responsible for infection, possibly due to an insufficient amount of DNA or the occurrence of a double infection.

All over Italy, the economic value of an old sheep (higher risk category for the presence of cysts) is very low, and it almost forces farmers to practice home slaughtering. According to our survey, the home slaughter practice was almost equally performed in different size farms, and it was not statistically dependent on the flock size. On the contrary, the prevalence of tapeworms was highly related to the number of dogs, as the probability to have taeniids into a farm had a 35% increase for each dog unit growth. Therefore, a rationalization of the number of dogs in the farm would be desirable in order to control tapeworm infection. Despite that swine home slaughter is subjected to veterinary inspection (regulated by law DL 333/98) (59), small ruminant home slaughter still requires a specific regulation, being a major risk control point for zoonotic parasitic diseases.

CONCLUSION

The present descriptive study highlights the importance of shepherd-dog parasites for public health. Data reporting working-dog parasites are lacking in the scientific literature, and the few published papers are specifically focused on *E. granulosus*. The homogeneity of the area, its own pastoralist vocation, and the high amount (648) of dog fecal samples analyzed from 50 farms offer a reliable picture of the area. The diagnosis of potentially zoonotic helminths as *Taenia multiceps* and *Toxocara* spp. should not be underestimated; and a health care of shepherd-dogs, following ESCCAP guidelines, is needed. Although this study has been carried out in an area where the pastoralism is traditionally advanced, the higher frequency of parasitism in shepherd-dogs compared with companion ones (1) does prove insufficient attention towardz dog health and welfare issues and suggests a lack of veterinary support. Indeed, regardless of the parasite involved, the occasional treatments in dogs, using sheep drugs, do not show real effectiveness. Small ruminant breeding has represented an important economic pillar in the whole Mediterranean Basin for ages; thus, it is time that public and private veterinarians cooperate for a pastoral upgrading all over the entire context.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BM, AM, RG, and GP have thought the project and collected and analyzed the samples. PD, CA, and FG have performed the molecular analyses. All Authors have written and revised the manuscript.

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Cryptosporidium Infections in Africa—How Important Is Zoonotic Transmission? A Review of the Evidence

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Cryptosporidium, a protozoan parasite in the phylum Apicomplexa, is the etiological agent of cryptosporidiosis, an intestinal infection characterized by profuse watery diarrhea. Over 30 species of *Cryptosporidium* are recognized, some host specific whereas others infect a broader host range. *Cryptosporidium hominis* and *Cryptosporidium parvum* are the species most commonly associated with human infection; *C. hominis* is largely associated only with human infections, but *C. parvum* is also associated with infection in animals, especially young ruminants. In some regions, cryptosporidiosis is a serious veterinary problem, particularly for calves, and lambs. Many outbreaks of human cryptosporidiosis have been associated with zoonotic transmission following contact with infected animals. In Africa, where cryptosporidiosis is a major contributor to pediatric morbidity and mortality, evidence suggests transmission is principally anthroponotic. Given the frequent close contact between humans and animals in Africa, the apparent predominance of human-to-human transmission is both interesting and puzzling. In this article, after a brief "text book" introduction to the parasite, we consider in separate sections the different aspects of relevance to *Cryptosporidium* transmission in African countries, describing different aspects of the various species and subtypes in human and animal infections, considering livestock management practices in different African countries, and looking for any characteristic "hot spots" where zoonotic transmission has apparently occurred. Studies where transmission networks have been investigated are particularly relevant. Finally, in a separate section, we try to gather these different strands of evidence together in order to assess the reasons behind the apparent predominance of anthroponotic transmission in Africa. Reviewing the available evidence provides an opportunity to re-think transmission pathways, not only in Africa but also elsewhere, and also to pose questions. Does the predominance of human-to-human transmission in Africa reflect a relative absence of zoonotic *C. parvum* in African livestock? Are Africans less susceptible to zoonotic

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Cryptosporidium infection, perhaps resulting from early immunostimulation by *C. hominis* or due to inherent genetic traits? Is the African environment—in all its variety—simply more detrimental to oocyst survival? Will the so-called hypertransmissible subtypes, currently relatively rare in Africa, be introduced from Europe or elsewhere, and, if so, will they fade out or establish and spread? Our intention with this manuscript is not only to summarize and consolidate diverse data, thereby providing an overview of data gaps, but also to provide food for thought regarding transmission of a parasite that continues to have a considerable impact on both human and animal health.

Keywords: Africa, anthroponosis, *Cryptosporidium*, epidemiology, subtype, transmission, water, zoonosis

INTRODUCTION

Addressing the Zoonotic Transmission Enigma

When *Cryptosporidium* was first discovered it was considered primarily as a parasite of animals, with the first human cases not identified until some 70 years later. The importance of *Cryptosporidium* as a pathogen was first really understood in the subsequent decade, and, at this time, transmission was considered to be largely zoonotic. Anthroponotic transmission was soon recognized, and, with the advent of more precise molecular tools, it became clear that there was a multiplicity of species and genotypes with different host-specificities. However, despite frequently being described as "ubiquitous," there are clearly geographical differences in the distribution of species, genotypes, and transmission routes. We are now more acutely aware of the global disease burden due to cryptosporidiosis, with the brunt of that burden borne by young children in African countries. At the same time, we have the enigma that, despite the closer relationship between people and animals in African countries compared with more industrialized countries, zoonotic transmission seems to occur less frequently in Africa (and some other regions) than in more developed regions, such as Europe.

In this article we explore this further, first giving a general introduction to the parasite itself, then providing a background on the parasite as a zoonosis and some background information on the burden from cryptosporidiosis in Africa, concerning both human and animal health. An overview of the species and subtypes of *Cryptosporidium* identified in infections in African countries is provided based on published papers, and also current perspectives on the potential for waterborne transmission. It should be noted, however, that identification of species and subtypes of *Cryptosporidium* is reliant on molecular techniques, which, in turn, require a relatively sophisticated laboratory with steady electricity supply and reagents that must be transported, and stored, frozen. In many parts of Africa, the infrastructure for molecular characterization is not yet developed and this means that our insights are, likewise, patchy.

We then consider animal husbandry in African countries, with emphasis on cattle, the species most associated with zoonotic transmission elsewhere. We tie this to an overview of those places and situations in Africa where zoonotic transmission has apparently occurred and try to identify defining characteristics. Finally, we extract from the previous sections those issues that are relevant regarding possible reasons why zoonotic transmission may occur less frequently in African countries than elsewhere, and compare and discuss their likely effects on transmission routes. In addition, we discuss whether, on both a global basis and from the African perspective, there is likely to be a shift toward an increase or decrease in zoonotic and anthroponotic transmission.

General Introduction to *Cryptosporidium* and Cryptosporidiosis

Cryptosporidium is a unicellular parasitic protozoan in the phylum Apicomplexa. Although considered a member of coccidia, evidence indicates that it has a closer affinity with gregarines, a large group of Apicomplexa considered particularly primitive (1). This classification has implications for the survival and spread of this parasite. To date, over 30 species of *Cryptosporidium* have been identified, some of which are host specific, whereas others are more promiscuous regarding host infectivity. Furthermore, whereas infection with some species of *Cryptosporidium* tend to be associated with little or no illness, others are particularly pathogenic with severe symptoms, which may even result in mortality. However, whether infection manifests as disease (cryptosporidiosis), and the severity of that disease, also depends on host factors, particularly those associated with host immunity and other health challenges.

Cryptosporidiosis usually manifests as a gastrointestinal disease, with diarrhea the most common clinical presentation. The lifecycle is predominantly fecal-oral, although often indirect with transmission by a vehicle such as water or food (see **Figure 1**). Although this article focuses on gastrointestinal infection, it should be mentioned that, for some *Cryptosporidium* species and hosts, respiratory cryptosporidiosis is also relevant. The oocyst transmission stage, which is infectious upon excretion without any requirement for maturation in the environment, is very robust and can be shed in high quantities, both characteristics that facilitate transmission via environmental contamination. When an infective oocyst is ingested it excysts in the small intestine and the released sporozoites invade the epithelial cells, where, in an epicellular location (intracellular but extracytoplasmic), asexual multiplication occurs. The resulting merozoites invade neighboring cells, and sexual multiplication occurs with the production of microgamonts and macrogamonts; following fertilization of the macrogamonts,

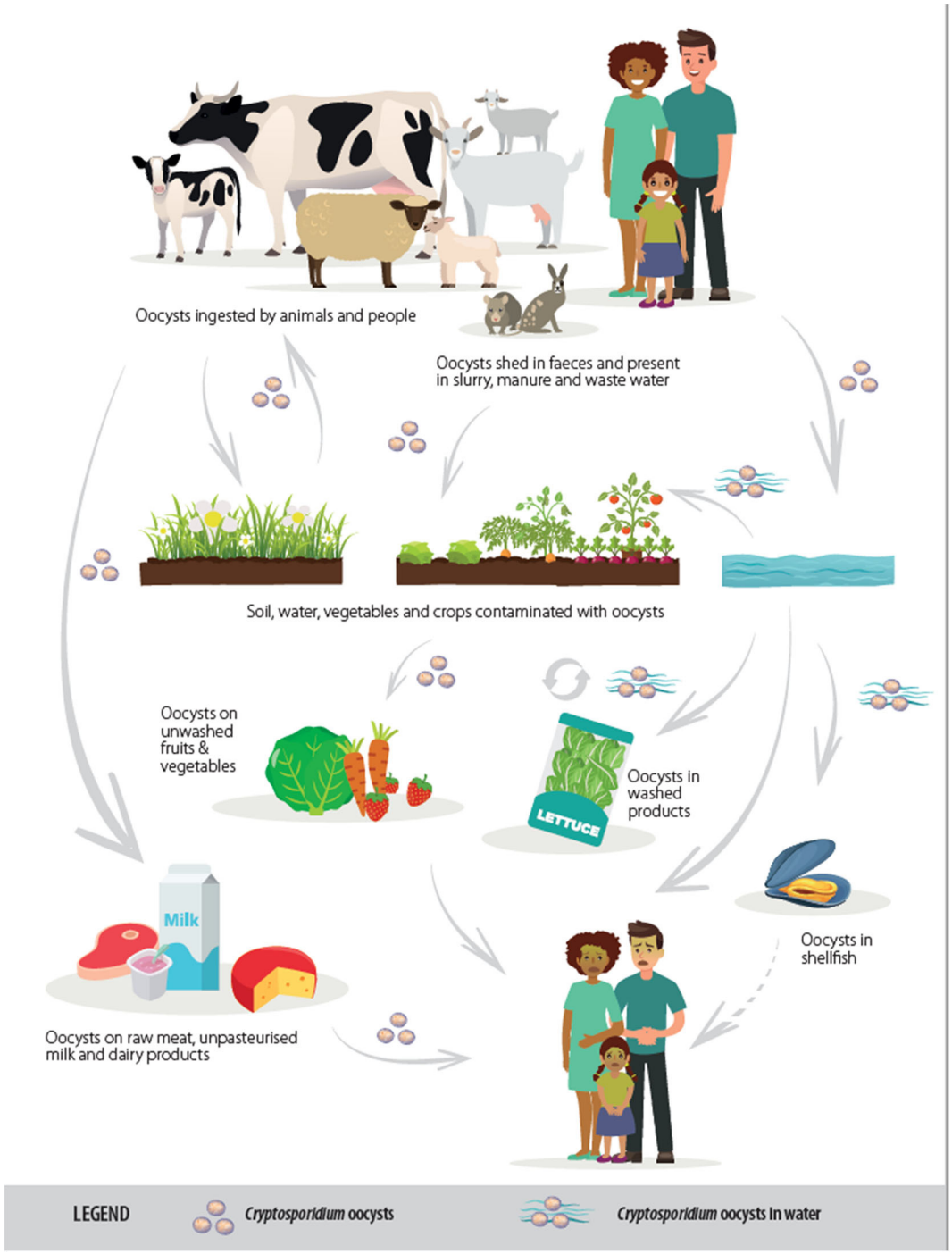

FIGURE 1 | Transmission routes of *Cryptosporidium* spp. [from (2), copyright European Food Safety Authority].

oocysts are produced that sporulate within the host before being shed in host feces.

The pathogenesis of cryptosporidiosis is associated with damage and changes to the cells of the intestinal wall; the interaction of *Cryptosporidium* sporozoites and merozoites with host cells results in signaling cascades with molecules (such as proteases and hemolysins) damaging cells, increasing fluid secretion, and causing malabsorption. Although usually self-limiting in the immunologically robust host, post-infection sequelae have been reported in human cases, although may reflect host immune responses or gut dysbiosis, rather than infection *per se*.

Cryptosporidium was first identified as an infection of animals, with human infections not reported until the mid-1970s. Even by 1980 only a handful of human cases had been reported, and, as a primary cause of acute diarrheal disease, it was largely unrecognized until the global HIV pandemic emerged, when *Cryptosporidium* became one of the first defining entities of AIDS. At this point, the potential for large communitywide outbreaks of waterborne cryptosporidiosis was also recognized, with various sizeable waterborne outbreaks documented.

For many years, only a single species, *C. parvum*, was really noted as the cause of human cryptosporidiosis, with *C. hominis* not recognized as a separate species until 2002 (3). Among the 30 or so *Cryptosporidium* species now identified, *C. parvum* is considered of substantial veterinary relevance to young livestock (calves and lambs), being considered as one of the most important causes of neonatal enteritis in young ruminants globally (4), and is also considered of major importance as a zoonotic species. Other zoonotic species include *C. meleagridis* (also commonly found in poultry), *C. cuniculus* (found in rabbits), and *C. ubiquitum* (commonly found in sheep); *C. hominis* largely infects humans. Other species are also generally host specific.

In people, where most infections are caused by *C. hominis* and *C. parvum*, the disease is generally acute and self-limiting, with symptom onset within about a week, and causes prolonged or persistent diarrheal episodes more often than other enteric pathogens. However, as treatment options are limited (the only FDA licensed treatment, nitazoxanide, licensed for treating patients aged 1-year and older, is only considered effective in those with healthy immune systems), it can be a serious illness in the very young, malnourished, and immunocompromised. In low-income countries, cryptosporidiosis is a major cause of infectious-disease mortality in children below 2 years (5).

We are now well aware of the multiplicity of species of *Cryptosporidium* and, in particular, the two main species infecting humans and the important differences in their epidemiology and transmission routes. As oocysts of *C. hominis* and *C. parvum* (and of most other zoonotic species) are morphologically identical, determining the infecting species relies on use of molecular tools. Such techniques are now used in many studies to determine species, and, often, subtypes, in *Cryptosporidium* infections, both in humans and animals, and have provided some insights into distributions and risk factors. However, many publications, even recent, refer to human *Cryptosporidium* infection as being with *C. parvum*, even when the species has not actually been determined. In many African countries, the infrastructure and trained personnel for such molecular analyses to determine species are not yet in place and this is reflected in the information available.

Cryptosporidium As a Zoonotic Agent: A Historical Perspective on Species and Genotypes

As noted, the first infections with *Cryptosporidium* were identified in animals, and many subsequent reports in the early 1980s concentrated on infections in various animals, with particular emphasis on livestock and rodents. The two first recorded human cases of cryptosporidiosis were from people living on farms (6, 7), and although the likelihood that cattle may have been the infection source was not raised, these cases strengthened the supposition that cryptosporidiosis is primarily an animal infection, with zoonotic potential.

Although cross-infection studies from *Cryptosporidium* isolates from guinea pigs failed to infect other animal species (8), further cross-infection studies with isolates from calves resulted in infections being established in lambs, calves, pigs, rats, mice, guinea pigs, and chicks (9). The latter authors suggested that their success with cross-infection studies, compared with the lack of success of Vetterling et al. (8), reflected that in their own experiments the challenged hosts were less than 1-day old and specific-pathogen free. Based on their results, they went so far as to propose that *Cryptosporidium* could be a single-species genus, much like *Toxoplasma gondii* (9). In retrospect, it is easy for us to see that this confusion arose due to the latter cross-infection studies using *C. parvum*, and the first cross-infection studies using a host-specific *Cryptosporidium* species (presumably *C. wrairi*). However, further experimental infection studies also supported the hypothesis that *Cryptosporidium* lacks host specificity and should therefore be regarded as a potential zoonosis (10). The first description of cryptosporidiosis in a veterinary student was published in the same year (11) and has been followed by at least a dozen more such reports since then.

Although a review of the taxonomy of *Cryptosporidium* in 1984, did not support the view of a single species, it concluded that although 19 species had been named at that time, only 4 should be considered valid: *C. muris* in mammals, *C. meleagridis* in birds, *C. crotali* in reptiles, and *C. nasorum* in fish (12). One year later, *C. parvum* was proposed to be the *Cryptosporidium* species infecting most mammals, including humans, distinct from *C. muris* for which the reported oocyst size was larger (which Levine had apparently overlooked) (13).

Although cryptosporidiosis as a zoonosis was rapidly accepted, at around the same time various studies reported that not all human infections were associated with animal contact. A study in a British children's hospital noted that most patients infected with *Cryptosporidium* did not have a history of close animal contact, suggesting that person-to-person transmission was as important as zoonotic transmission (14). Similarly, a UK survey of patients with gastrointestinal symptoms found that of the 5% with cryptosporidiosis, contact with animals was not a feature (15). Indeed, an extensive long-term study from Wales (16), culminated with the authors concluding that although animals may be a source of *Cryptosporidium* infection in people, human-to-human infection probably occurs more commonly, and cryptosporidiosis should not be regarded primarily as a zoonosis.

Most of these first epidemiological studies exploring *Cryptosporidium* infections, uncovering evidence of both zoonotic and anthroponotic transmission, are from industrialized countries, particularly Europe and North America. However, among these earlier reports are two from Liberia that describe associations of *Cryptosporidium* infections in children under 5-years of age with a range of different

factors (17, 18). As with the reports from UK (15, 16), the authors of the Liberian study concluded by questioning the general belief of that time, that cryptosporidiosis is primarily a zoonosis. They grounded their suspicion on their findings that cryptosporidiosis in Liberian children seemed to occur in regions where domestic animals were uncommon, was associated with household crowding and bottle feeding, and that peak prevalence was among infants still carried on their mothers' backs and thus not in particularly close contact with animals or the wider environment (17, 18). Thus, although no suggestion was made that more than one species of *Cryptosporidium* may be involved in gastrointestinal cryptosporidiosis in humans, it was recognized from studies, including in Africa, that different epidemiologies could be important, and that there could be a role for both animal-to-human and human-to-human transmission.

It was not until the late 1980s and early 1990s that evidence began to mount, based initially on isoenzyme analysis and thereafter molecular tools, such as PCR-RFLP and sequence investigation, that, as well as there being an animal-to-human *or* a human-to-human cycle of *C. parvum* infections, there was also another type of *Cryptosporidium* that essentially infected solely humans. These two groups were initially designated as the zoonotic "cattle" genotype (usually designated genotype II or sometimes genotype C) and the anthroponotic "human" genotype (genotype I or genotype H); this latter type of *Cryptosporidium* received a formal species designation, *C. hominis*, in 2002 (3). It is now well established that not only are there two species of *Cryptosporidium* causing most cases of human cryptosporidiosis, *C. parvum* with its two potential cycles of animal-to-human or human-to-human and *C. hominis* being almost exclusively human-to-human, but that there are also subtypes within these species that also seem to have virulence and host infectivity differences. Indeed, some *C. parvum* subtypes (e.g., IIc, IIe, and IIm) are apparently almost exclusively limited to human infections, despite the species being generally considered zoonotic. Indeed, *C. parvum* subtype IIc has recently been proposed as being classified as an anthroponotic subspecies - *C. parvum anthroponosum* (19). Whereas PCR and sequencing at the SSU rRNA gene is now the most common method for determining *Cryptosporidium* species, for determining subtype within species most reports use sequence variations in part of the hypervariable 60 kDa glycoprotein (gp60) gene; use of these markers has been described in several publications [e.g., (20–22)]. These molecular tools provide not only a means of exploring transmission pathways in greater detail, but are also useful in outbreak investigations; an outbreak of waterborne cryptosporidiosis in which *C. hominis* is identified in those infected will point investigators toward considering sewage contamination, rather than runoff from agricultural land.

THE IMPACTS OF CRYPTOSPORIDIOSIS IN AFRICA

Human Health Impacts

There is no doubt that cryptosporidiosis has a substantial health impact globally, particularly in lower-income countries. Most African countries are classified using World Bank definitions (23), as having low-income or lower-middle income economies, with the exception of Algeria, Botswana, Equatorial Guinea, Gabon, Libya, Mauritius, Namibia, and South Africa, which are classified as upper-middle income, and Seychelles being high income. Of the 31 countries globally classified as being in the lowest income group, 24 (77%) are in Africa.

One of the earliest studies investigating the impact of *Cryptosporidium* in an African country was from Guinea Bissau, and demonstrated that *Cryptosporidium* was associated with excess mortality in children younger than 12 months, with this excess mortality persisting into the second year of life (24). Although this impact from cryptosporidiosis in particular countries has long been assumed, the first comprehensive data demonstrating this were produced relatively recently, from the Global Burden of Disease (GBD) and the Global Enteric Multicenter Study (GEMS) outputs [e.g., (5, 25, 26) etc.]. These studies provided the first global estimates on impacts of cryptosporidiosis (among other diseases) in different age groups and different countries, in terms of mortality, morbidity, and disability-adjusted life-years (DALYs). A meta-analysis published in 2018 showed that earlier reports probably under-estimated the true burden by not taking into account impacts occurring after the acute phase of infection, such as decreased growth, particularly weight gain, and a greater risk of subsequent episodes of infection (27). As *Cryptosporidium* diarrhea damages gut endothelial cells and microvilli, absorption of macronutrients, and micronutrients are impaired (28, 29). In addition, *Cryptosporidium*-related malnutrition results in secondary impairment of cell-mediated immunity, which is associated with increased susceptibility to other infectious diseases. Other long-term sequalae include reduced cognitive development, poor school performance, and elevated risk of cardiovascular and metabolic diseases later in life (30, 31), all likely to have a disproportionate effect on the global poor.

Cryptosporidium infection in children under 5 years was estimated to be associated with 44.8 million diarrheal episodes and 48,300 deaths globally (27). Of these, the vast majority were from Africa, accounting for 75% of the diarrheal episodes and 88% of the deaths (27). In particular, the burden of *Cryptosporidium*-associated diarrhea is greatest in Sub-Saharan Africa, especially Nigeria and the Democratic Republic of the Congo (DRC) where about 48% of the under-5 associated deaths occur (27). When including downstream effects of growth shortfalls associated with cryptosporidiosis, it was estimated that the burden of this parasite could be 2.5 times higher than previous estimates (27), and recognized that accounting for the direct or indirect burden of asymptomatic infections could elevate these estimates even further.

Veterinary Health Impacts

It is well known that whereas infection with some species of *Cryptosporidium* has apparently marginal impact on host health, ruminants, particularly young animals, infected with *C. parvum* may suffer from profuse watery diarrhea, inappetence, lethargy, and dehydration; it is not unusual for death to occur, particularly in neonates. With an infectious dose for neonatal calves as low as

17 oocysts (32), ensuring that young stock are not exposed to an infectious dose on farms where other stock are already infected can be challenging. As with humans (see previous subsection), it has also been shown that severe cryptosporidiosis in calves and lambs may have long-term consequences regarding growth, weight gain, and productivity (33–35), as well as the more immediate effects from the acute infection.

Cryptosporidiosis outbreaks on farms are not commonly investigated and reported, but have been described among cattle and goats from farming enterprises in Europe and Asia [e.g., (36–38)]. Although there are no published reports of cryptosporidiosis outbreaks among livestock in Africa, several studies from different African regions have reported on calf diarrhea without any clear attribution to a specific etiological agent (39–41). Given that a variety of etiological agents, as well as *Cryptosporidium*, can cause calf diarrhea (e.g., rotavirus, coronavirus, bovine viral diarrhea virus, *E. coli*, *Clostridium perfringens*, *Salmonella* spp., and coccidia such as *Eimeria zuernii* and *E. bovis*), these data do not necessarily indicate cryptosporidiosis. A similar situation applies to lambs and goat kids.

A systematic review of *Cryptosporidium* infections in livestock (42) noted the prevalence being highest in the Americas and Europe—and commented that under-investigation in particular regions was not the reason for this skewed distribution. However, publication bias and insufficient information may have excluded some relevant studies (42). Nevertheless, some researchers report that *Cryptosporidium* oocysts are frequently detected in diarrheic calves in different African countries (43–46), but usually without determining whether cryptosporidiosis is the cause of the symptoms. Many of these studies use modified Ziehl-Neelsen (mZn) for identification; this has low sensitivity and specificity, and does not enable identification of the *Cryptosporidium* species. Studies using more accurate tests have revealed contrasting results; for example, a study from Tanzania used, in addition to mZn, immunofluorescent antibody testing (IFAT), auramine phenol staining, and molecular methods to investigate calves for *Cryptosporidium* infection and, using the latter methods, did not detect *Cryptosporidium* shedding in 943 calf samples, of which over 6% were diarrheic, despite some positive results with mZn (47). The authors suggest that data obtained using mZn should be treated with caution. In contrast, a study in Egypt using molecular tools reported a 32% *Cryptosporidium* prevalence in cattle (48). Two studies from Ethiopia, both of which used molecular methods for determining infecting species, provide contrasting data: a study from two large dairy farms in central Ethiopia showed that *Cryptosporidium* infection was common (40% cumulative incidence), with *C. parvum* most common in pre-weaned calves and *C. andersoni* in post-weaned calves (49). In contrast, an earlier study in an overlapping area, included 449 calves from both smallholder farmers and dairy farms and detected less than 10% infection, with *C. andersoni*, *C. bovis*, and *C. ryanae* identified, but not *C. parvum* (50). It is noteworthy that whereas the first two studies (48, 49) associated *Cryptosporidium* infection with a calf-health impact, the latter (50) reported that the calves were generally healthy, with only a few cases of watery diarrhea.

CRYPTOSPORIDIUM INFECTIONS IN AFRICA: DISTRIBUTIONS OF SPECIES/GENOTYPES

Two reviews of *Cryptosporidium* in Africa were published relatively recently (51, 52), the latter of which also considers *Giardia*. Although these articles have slightly different overall perspectives, both contain information on molecular epidemiology and have assimilated data from the literature describing the occurrence of different *Cryptosporidium* species/genotypes in various host species in different regions. Aldeyarbi et al. (51) used a defined literature search to gather data, but the authors excluded studies considered to be weakly designed or biased (although how these criteria were determined is unclear). The conclusion from this review is that both anthroponotic and zoonotic transmission cycles have potential for infecting people in Africa, that infections in wild animals are "essential contributors" to environmental contamination that threatens human health, but that *C. hominis* was the predominant species infecting people in many studies, regardless of host immune status (51). In addition, the authors noted that among human *C. parvum* infections in various sub-Saharan African countries, the GP60-subtype family IIc [previously Ic, proposed now as *C. parvum anthroponosum*; (19)] often predominates. As noted, this is a human-adapted subtype occurring almost exclusively in human infections. A recent systematic review and meta-analysis investigating geographical distribution of this subtype in human infections (53) found its occurrence was associated with countries with low GDP per capita and poor sanitation; of 81 relevant single-country articles included in their study (20 from Asia, 20 from Africa, 21 from Europe, 7 from North America, 3 from South America, 10 from Australia/Oceania), 35 reported the presence of *C. parvum* IIc subtype (*C. p. anthroponosum*), of which 14 were from Africa (53). Thus, we can extrapolate a significant association between this particular subtype and Africa, as compared with the rest of the world combined ($p < 0.0086$). Furthermore, the proportion of this subtype among *C. parvum* isolates in those studies reporting its presence was also higher in Africa (2–100%; mean 75%, median 76%) compared with the rest of the world (3–100%; mean 36%, median 22%).

In a study based on the GEMS data, but looking specifically children younger than 2 years, in some regions of sub-Saharan Africa and south Asia, of 28 *C. parvum* infections from Mali ($n = 13$), Kenya ($n = 9$), Mozambique ($n = 5$), and Gambia ($n = 1$), all were anthroponotic IIc or IIe (54). In industrialized countries, subtype IIaA15G2R1 predominates among both dairy cattle and human *C. parvum* infections, and is sometimes described as hypertransmissible (55, 56). However, in Africa, this subtype has been reported from both cattle and humans in only two countries, Egypt and Tunisia, and also in people in Nigeria—indicating that it does not (yet) predominate here. Thus, despite presently predominating in some circumstances and regions, whether it always or intrinsically transmits more successfully has not been clearly demonstrated.

A relatively high frequency of *C. meleagridis* infections has been reported in African studies, as mentioned by Aldeyarbi

et al. (51) (of the studies considered, the authors report *C. meleagridis* among 21% of the immunocompromised and 10% of non-immunocompromised people, compared with below 1% in the developed world). Although *C. meleagridis* is also a zoonotic *Cryptosporidium* species, many of the papers from Africa do not indicate an association with infected animals or birds [although an association with chicken *C. meleagridis* infections has been suggested in Côte d'Ivoire (57) and Nigeria (58)] and some actually indicate a lack of association with chicken infections [e.g., (59)], perhaps suggesting transmission from a human source.

The more-recent review paper (52) tabulates *Cryptosporidium* infections in papers from African countries published from 2010 to 2016 according to prevalence (occurrence) in human cohorts (along with information on diagnostic technique), and by *Cryptosporidium* species and genotype according to patient group. Similar data for animal hosts, both wildlife and domestic animals, are tabulated separately. This is a comprehensive undertaking (although data published separately from the same research studies are sometimes listed twice), and, similarly to Aldeyarbi et al. (51), the authors note the predominance of anthroponotic transmission, with *C. hominis* and anthroponotically transmitted *C. parvum* being reported principally in human infections.

Using a literature survey of PubMed (using the search terms of each African country in turn along with Boolean AND and cryptosporidi*) we identified a further 45 papers not included in the Squire and Ryan (52) review, 34 of which had been published from 2017 and onwards (**Figures 2**, **3**). Of these additional articles, 17 involved *Cryptosporidium* in human hosts only, 19 involved animal hosts only, and 9 considered both human and animal hosts. One article was from the Gambia (60), a country not featuring in the Squire and Ryan (52) review. This article reported more closely on *Cryptosporidium* infections in children enrolled in the GEMS study, and, although most (>80%) were *C. hominis*, a significant association with animals (cats or cows) living in the compound was also reported (60). However, given the host specificity of *C. hominis*, and that these animals were not themselves tested for infection, the presence of animals in the compound could be an indicator of another risk factor, rather than being the infection source.

Of relevance regarding *C. hominis* infections in Africa, is that subtype IbA10G2, which is associated with most outbreaks in industrialized countries (22, 61–63), and has been described as being hyper-virulent (55, 61, 62), despite evidence for differences in clinical symptoms or advanced transmission within gp60 allele families being weak, seems to occur rarely in Africa. It was not found in a subtyping performed with the GEMS study samples (54), and has been reported only sporadically in surveys [from 3 children (of 28 with subtyped *C. hominis*) in Nigeria (64); from 1 HIV/AIDS patient (of 19 with subtyped *C. hominis*) in Ethiopia (65); a maximum of 2 children (of 19 with subtyped *C. hominis*) in South Africa (66); and 1 HIV and TB patient (of 2 typed with *C. hominis*) in Mozambique (67)].

Among the 32 articles investigating *Cryptosporidium* in animals in Africa included by Squire and Ryan (52), 16 (50%) reported on cattle and 4 reported on sheep or goats; in the additional articles that we identified, 8 reported on cattle and 7 on sheep and/or goats. These articles do not indicate any clear patterns regarding infectious species or subtypes, although one with data from cattle reported the presence of the "hyper-transmissible" IIaA15G2R1 subtype (68); however, as reported from other global regions, the data indicate that younger animals (both calves, lambs, and goat kids) seem more likely to be infected with *C. parvum* than older animals (69). Nevertheless, age does not seem to be the only determinant regarding infection with zoonotic *Cryptosporidium* species in cattle husbandry. For example, a Zambian study investigating the species of *Cryptosporidium* in calves demonstrated that whereas calves from intensive dairy farms and extensive commercial beef farms (mean calf age 15 and 26 days, respectively) were largely infected with *C. parvum*, among calves infected with *Cryptosporidium* on small traditional farms (mean calf age 22 days), only *C. bovis* was identified (70).

Among small ruminants, the occurrence of *C. parvum* infection seems to be rather low in studies from Africa; among the 6 studies compiled by Squire and Ryan (52), of those including small ruminants (sheep, goats), only 3 (50%) reported the presence of *C. parvum*, with the other studies reporting only *C. xiaoi*. Of the more recent papers, a couple with very low numbers of samples (between 1 and 8 samples) report *C. parvum* in sheep and goats [e.g., from Nigeria (58); from Sudan, (71); from Uganda, (72)]. Larger studies, however, indicate that *C. parvum* occurs relatively infrequently in African small ruminants, with *C. xiaoi* predominating in sheep and goats in Ghana (73) and a study in Ethiopia including 389 lambs under 5 months of age found only *C. ubiquitum* (74). Although *C. ubiquitum* has zoonotic potential (75), human infections in Africa have been reported only extremely rarely [one study from Nigeria, with two publications noted by Squire and Ryan, (52)]. Of interest is that the single *C. parvum* isolate from a goat in the study from Ghana was typed as being the anthroponotic IIc subtype (*C. p. anthroponosum*); with molecular methods being the only analytical tool used, it is possible that this single *C. parvum* infection among 285 goat samples of which 95 were positive, represents carriage rather than infection. Furthermore, a comprehensive contact-network analysis study conducted in 4 African countries [Gabon, Ghana, Madagascar, and Tanzania; (76)], not only reported that *C. hominis* predominated among human isolates (from children below 5 years), but also that *C. hominis* occurred not infrequently in their animal contacts (goats, sheep, cows, dogs). However, given that these animals may well have ingested feces of infected children, it is unclear whether we can infer zoonotic transmission here rather than carriage in those animals in which *C. hominis* DNA was detected (12 cows, 5 goats, 1 sheep, and 3 dogs), given how rarely this species has previously been identified in animal infections. Indeed, the authors themselves emphasize that human-to-human transmission appears to be the predominant route in their 4 study sites, with zoonotic transmission contributing only marginally (76). It is unfortunate that environmental samples were not analyzed in this study, as this could, potentially, have added even greater weight to the study findings.

In addition to the two review papers concerned with *Cryptosporidium* in Africa (51, 52), a slightly older review considers *Cryptosporidium* in the Arab world (77), which

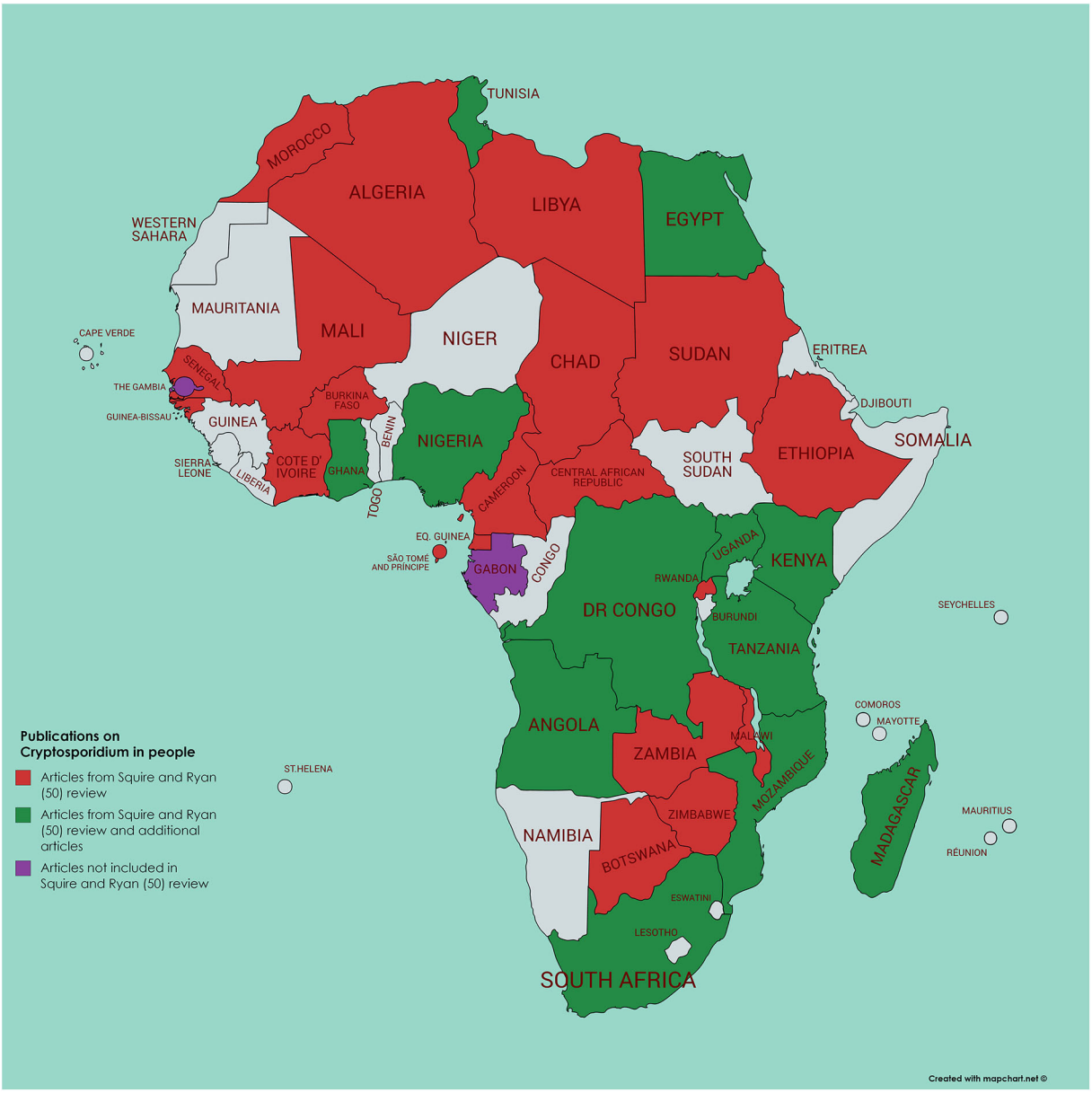

FIGURE 2 | Map of Africa indicating those countries where human infection with *Cryptosporidium* has been investigated; articles either referenced in Squire and Ryan (52) or identified by current literature search.

also includes some countries from Africa (specifically Algeria, Comoros, Djibouti, Egypt, Libya, Morocco, Mauritania, Somalia, Sudan, and Tunisia). Although the authors concluded that zoonotic transmission is important, little supportive molecular evidence was presented.

WATER AS A *CRYPTOSPORIDIUM* TRANSMISSION VEHICLE IN AFRICA AND POTENTIAL FOR CONTAMINATION

The potential for water to be a transmission vehicle for *Cryptosporidium* is accepted globally, with communitywide outbreaks and smaller outbreaks reported from multiple countries. The dearth of such outbreaks being reported from Africa probably represents limitations in technological capabilities and surveillance systems (78). It must also be considered that with a high background prevalence of diarrheal disease (of whatever etiology)—albeit varying regionally (79)—it is probably more difficult for an outbreak to be identified in Africa unless extremely dramatic. Even in countries with a relatively low incidence of diarrheal diseases and well-developed reporting systems, identifying a cryptosporidiosis outbreak may not be straightforward; not all cases seek medical attention, doctors may not request stool samples, and stool samples may

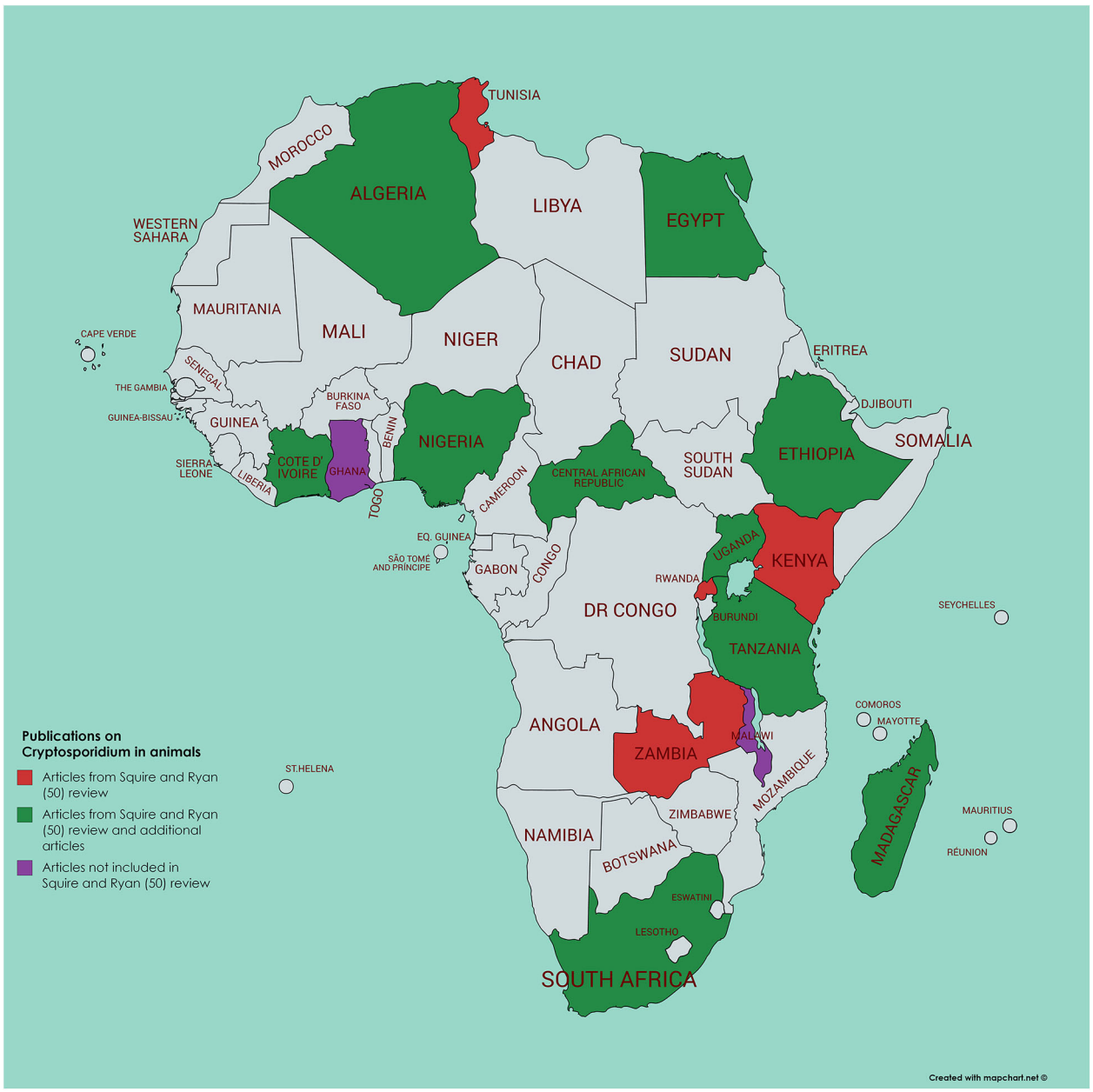

FIGURE 3 | Map of Africa indicating those countries where animal infection with *Cryptosporidium* has been investigated; articles either referenced in Squire and Ryan (52) or identified by current literature search.

not be analyzed appropriately (80, 81). Thus, lack of reporting of cryptosporidiosis outbreaks does not necessarily mean they do not happen. In order to improve our understanding of whether such outbreaks occur, ensuring the etiology of diarrhea in African countries is diagnosed and reported is probably the best place to start, rather than analyzing water samples. Although such analyses provide clues, the procedures are expensive and result interpretation may be difficult.

A review (82) identified 60 papers addressing *Cryptosporidium* in water in Africa. However, from the information presented it is difficult to extrapolate how many were concerned with surveillance of drinking water, the analytical methodology used, and the results obtained. By means of a literature survey of PubMed (using the search terms of each African country in turn along with Boolean AND and cryptosporidi* AND water) we identified just 21 papers from Africa (originating from 8 countries) for which drinking water (of different types) had been analyzed for contamination with *Cryptosporidium* (see **Figure 4** and **Supplementary Table 1**). Of these, 8 had been published after the Ahmed et al. (82) review, so would not have been included there; of the 13 remaining, 5 were cited by Ahmed et al. (82). Of relevance is that many of these 21 papers used methods

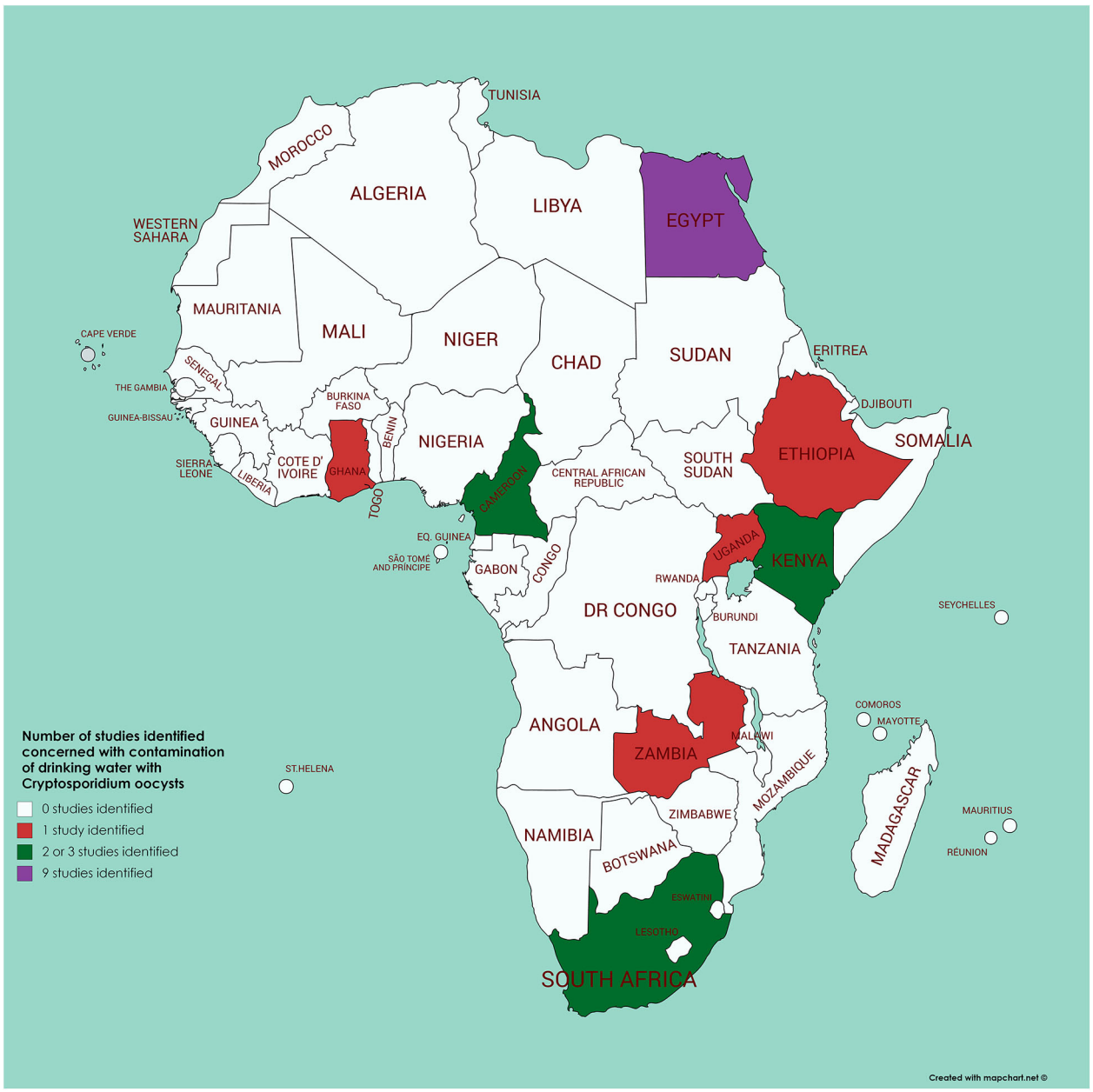

FIGURE 4 | Map of Africa indicating those countries where contamination of drinking water with *Cryptosporidium* has been investigated (see **Supplementary Table 1** for further details).

that seem unlikely to provide convincing results (e.g., sample volumes as low as 10 ml, with minimal processing steps, and mZn for detection of oocysts). Nevertheless, it is these articles that often provide data indicating the highest proportion of samples considered positive (over 40%), while papers using standardized methods for water analysis (those of ISO or US Environmental Protection Agency) tend to report lower proportions of positive samples [e.g., the work of Morris et al. from Kenya (83), Kifleyohannes and Robertson from Ethiopia (84), or Potgjeter et al. from South Africa (85)]. This suggests that the lack of specific detection techniques used in other studies may have resulted in false positive results. In addition, some of the studies using non-specific methods report extremely high concentrations of oocysts (tens or hundreds per liter), which may also indicate false positives (or excessively high contamination). None of the studies report subtypes of *Cryptosporidium* in the water samples, but 10 reported species, all of which mention *C. parvum*, with one of these from Egypt also noting the predominance of *C. hominis* (86) and one from Kenya reporting 6 samples with *C. parvum* and 3 containing *C. andersoni* (87). In this Kenyan study, the

samples in which *Cryptosporidium* was detected were from water with likely animal contamination, as cattle were watered there and elephants were known to use it (87). However, the evidence of animals being the source of the contamination is weak. Among the other 8 articles in which *C. parvum* was reported from the water samples, one [from the same region of Kenya as that of Muchiri et al. (87)], reports *Cryptosporidium* in one of 14 samples, with *C. parvum* detected (88). However, the other 6 articles (published between 1997 and 2019), although reporting *C. parvum*, did not apparently undertake any molecular analyses, and the species definition appears to be based upon supposition rather than results (**Supplementary Table 1**).

Two recent articles from the same research project used a modeling approach to consider *Cryptosporidium* contamination of rivers globally (89) and disease burden due to *Cryptosporidium* in surface water in sub-Saharan Africa explicitly (90). Worth noting is that hot spots for river contamination were identified in Nigeria, Algeria, and South Africa, with human contamination (point sources) considered to dominate over contamination from animals (diffuse sources). However, in some African countries (e.g., DRC, South Sudan, Chad, Ethiopia) more diffuse sources may predominate, although contamination of river water with oocysts in these countries was also considered to be amongst the lowest globally (89). Using not only the modeled contamination data, but also information on the proportion of population using surface water as a drinking water supply, along with drinking water treatments, Limaheluw et al. (90) estimated the cryptosporidiosis burden due to oocysts in the surface-water drinking water supply in sub-Saharan Africa to be 1.6×10^6 DALYs. The highest number of DALYs per 100,000 of population were in Eswatini (1022.8), Mozambique (828.5), and Kenya (715.2), and the lowest in Senegal (1.3). The extremely high DALYs in south and south-east Sub-Saharan Africa were partly explained by higher estimates of life-years lost in people with HIV/AIDS (90).

RUMINANT LIVESTOCK PRODUCTION IN AFRICA AND POTENTIAL EFFECTS ON *CRYPTOSPORIDIUM* TRANSMISSION

Zoonotic transmission of *Cryptosporidium* is most likely to originate from domestic livestock (cattle, sheep, goats), although other animals, notably rabbits (*C. cuniculus*) and poultry (*C. meleagridis*), may also be important sources. It is therefore relevant to consider ruminant livestock production systems in Africa, particularly those in countries with large cattle populations (91), when considering the potential for zoonotic transmission of *Cryptosporidium*. Livestock production systems vary between and within continents and countries and are classified on the basis of different criteria and metrics (92). In Africa, classification of livestock production systems is complicated by a plethora of factors such as sociocultural, agro-climate, land use, livestock densities and levels of intensification. On the basis of agro-climate or how feed for the animals is produced, Africa is dominated by land-based production systems, predominantly "mixed, rain-fed" and "grazing" (93). The pastoral grazing system occurs mainly in arid and semi-arid areas and the mixed rain-fed system is common in the humid, semi-humid, and tropical highland areas, but also occurs in the arid-semiarid climatic zones (94). Grassland utilization ranges from total nomadism (no permanent place of residence, no regular cultivation) via semi-nomadism, transhumance, and partial nomadism, through to stationary animal husbandry (92). Ruminant livestock production can also be divided by intensity of production, from extensive to intensive. Extensive production ranges from small-scale, subsistence production (smallholder farms) dependent on mixed crop-livestock systems to large pastoral holdings that rely mainly on rangeland grazing. In contrast, intensive production system involves geographically-concentrated and commercially-oriented specialized production that may develop into industrialization, possibly involving multinational firms and contract farming (92). Although both intensive and extensive production of ruminant livestock occurs in Africa, extensive and pastoral systems tend to predominate. Of relevance is that calving rate is relatively low (overall about 60%) and calf mortality relatively high (around 20%) in the majority of management systems in Africa (94, 95) compared with rates reported from other countries. Similarly, for small ruminants, lamb and kid mortality risk is high, although the production rate is high and prolificacy between 1 and 1.5, lamb and kid mortality risks are equally high (94). These parameters, in association with year-round breeding, mean that neonate density tends to be relatively low in most production systems.

The largest sub-Saharan African livestock populations are in East Africa with 55.3% of the total livestock units, followed by West Africa, Southern Africa, and Central Africa, with 27.1, 9.4, and 8.2%, respectively (94). In arid and semi-arid zones, the dominant species are goats and sheep followed by cattle, whereas in sub-humid zones, cattle predominate followed by goats and sheep with the highest densities of livestock in the highlands (94). Two countries in East Africa, Ethiopia and Sudan, have far higher cattle populations than other countries in the region. An overview table of some relevant key figures regarding cattle production in some African countries (Burkina Faso, Egypt, Ethiopia, Kenya, Nigeria, Uganda) is provided (**Table 1**), in which the data have been extracted from the series of FAO publications concerned with livestock production systems (96).

Given the heterogeneous cattle-production systems, the varying lifestyles of human populations, and the climatic variability in Africa, the environmental load of *Cryptosporidium* oocysts and the risk of human infection from them probably vary significantly throughout the continent. In arid and semi-arid environments, where cattle are kept under extensive management systems in pastoral and agro-pastoral settings, large numbers of cattle graze together. Although this can potentially lead to high pasture contamination, it tends to be seasonal as pastoralists move their animals in search of pasture and water, and *Cryptosporidium* oocyst loads are expected only to be higher where these resources are available (97). In the pastoral cattle management system, cows with younger calves remain at home under the management of women and children on the limited food reserved for them, while other animals may travel long distances. Whether such division of labor

TABLE 1 | Relevant comparative data of different cattle-production systems in some African countries.

Country, dairy or beef. Size of national herd	**Production system**			
Burkina Faso (beef and dairy)	**Extensive pastoral**	**Extensive agro-pastoral**	**Semi-intensive**	**Intensive**
Total: 9 million	Proportion: 12–17% Size: 100-several thousand animals Breeds: Fulani Zebu	Proportion: 75% Size: 5–100 animals Breeds: local (taurine and zebu)	Proportion: 11% Size: 2–10 animals Breeds: not described	Proportion: 5% Size: 10–25 animals Breeds: not described
Egypt (beef and dairy)	**Extensive**	**Semi-intensive**	**Intensive (small-scale)**	**Intensive (large-scale)**
Cattle and buffalo Total: 8.1 million	Proportion: 30% Size: 1–10 animals Breeds: indigenous cattle and buffalo	Proportion: 60% Size: 10–50+ animals Breeds: improved local	Proportion: 7% Size: around 10 animals Breeds: exotic for milk and exotic and crossbreeds for beef	Proportion: 3% Size: 100–1,000s Breeds: exotic for milk and exotic and crossbreeds for beef
Ethiopia (beef and dairy)	**Pastoral/agro-pastoral**	**Mixed-crop livestock**	**Urban/peri-urban**	**Commercial**
Total: 56.7 million	Proportion: 14% Mostly dairy (some for sale for feed lot or used as draft oxen): Size: usually 10–20 animals, but large herds (>200) common Breeds: indigenous	Proportion: 77% Dairy: Size: 4 animals Breeds: indigenous Beef fattening: Size: 1–4 animals Breeds: indigenous Zebu	Proportion: 7% Dairy: Size: 5–10 animals Breeds: high-grade or crossbred animals Beef fattening: Size: 1–8 animals Breeds: indigenous Zebu	Proportion: 3% Dairy: Size: <30 =small, 30–100 = medium, >100 = large Breeds: purebred exotic, high-grade or crossbred dairy animals. Beef feedlot: Size: 100–1,500 Breeds: Borana
Kenya (dairy)	**Extensive**	**Semi-intensive**	**Intensive (small-scale)**	**Intensive (large-scale)**
Total: 4.2 million	Proportion: 15% Size: 10–50+ animals Breeds: exotic breeds and crosses of indigenous breeds.	Proportion: 45% Size: 1–20 animals Breeds: Mostly crosses and exotics breeds (42% Friesian, 25% *Bos indicus* (Zebu, Sahiwal, Boran), 18 % Ayrshire, 12% Guernsey, 3% Jersey)	Proportion: 35% Size: 1–20 animals Breeds: exotic high-grade dairy (Friesian, Ayrshires, Fleckvieh, Guernsey and Jersey	Proportion: 5% Size: >20 animals Breeds: exotic high-grade dairy (Friesian, Ayrshires, Fleckvieh, Guernsey and Jersey
Kenya (beef)	**Extensive pastoralism**	**Extensive ranching**	**Semi-intensive (agro-pastoralism)**	**Intensive (feed lot)**
Total: 14.3 million	Proportion: 34% Size: 50 animals Breeds: indigenous, mainly African Zebu, also Boran and Sahiwal	Proportion: 11% Size: 150 animals Breeds: improved Boran and exotic (Hereford, Simmental, Charolaise and Angus)	Proportion: 54% Size: 10–12 animals Breeds: mainly crossbreeds and pure exotic breeds	Proportion: 1% Size: 500–3,000 Breeds: Boran, Sahiwal and Zebu crosses; specialized beef breeds (Charolaise, Angus, Frisian)
Nigeria (dairy)	**Extensive/traditional (pastoral)**	**Semi-intensive (agro-pastoral)**	**Intensive (modern)**	**Commercially oriented**
Total: 18.2 million	Proportion: 82% Size: 100–300 animals Breeds: Indigenous (e.g. Bunaji, Gudali, etc.)	Proportion: 17% Size: 20–100 animals Breeds: Indigenous	Proportion: 1% Size: small scale =50–500; medium 50–1,000; large = over 1,000 animals Breeds: usually exotic, some indigenous	Proportion: Negligible
Uganda (beef)	**Pastoral/mixed smallholder**	**Agro-Pastoral**	**Semi-intensive**	**Commercial ranching**
Total: 11.4 million	Proportion: 90% Size: 100–300 animals Breeds: Mostly local (Ankole and local zebu)	Proportion: <10% Size: 10 animals Breeds: indigenous with some cross breeds (e.g., East African Zebu and Holstein Friesian and Ankole and Holstein Friesian	Proportion: <10% Size: 1–5 animals up to 20 Breeds: crossbreeds of East African Zebu and Holstein Friesian	Proportion: <10% Size: 500–3,000 animals Breeds: Indigenous, cross, exotic (often imported)

Information derived from: Food and Agriculture Organization of The United Nations (FAO): Africa Sustainable livestock 2050 http://www.fao.org/in-action/asl2050/resources/documents/livestock-production-systems/en/ (96).

and animal care among family members increases the risk of transmission of *Cryptosporidium* to young children requires further investigation.

By numbers of livestock, Ethiopia ranks first in Africa. The cattle production sector here is highly heterogeneous, comprising both traditional pastoral/agro-pastoral and mixed crop–livestock production systems and the market-oriented, intensive and specialized producers. Pastoral/agro-pastoral production dominates in the Ethiopian lowlands, where livestock are managed under pasture-based extensive systems. Cattle dominate the livestock population here, accounting for 25% of the national herd (**Table 1**). The mixed crop-livestock system of Ethiopia carries more than 70% of the cattle population, with extensive management systems and supplementation from crop residues [**Table 1**, (98)]. This system occurs often in densely populated regions where animals are frequently kept close to residential areas. Cattle manure here is used as fertilizer and for fuel as dried dung cakes, mainly prepared by women and children, potentially resulting in a high risk of exposure to *Cryptosporidium* oocysts (and other pathogens) (99).

Specialized commercial dairy systems (exotic and cross breeds) and feedlot (local zebu) operations in Ethiopia are concentrated in densely populated urban and peri-urban settings and constitute a very small fraction of the livestock population (98). However, in terms of environmental load of *Cryptosporidium* oocysts, these operations probably represent major hot spots in comparison with traditional or extensive livestock management. Interestingly, Aldeyarbi et al. (51) mention that most *C. parvum* infections seem to occur in urban settings in Africa, where, according to these authors, animals are not found close to residences. However, as described above, even in residential areas, people and animals often live in close proximity and Aldeyarbi et al. (51) may have under-estimated the extent of such urban-based farming in some areas of Africa.

Dairy cattle production in Kenya is the second largest contributor to the agricultural GDP and is classified into three production systems: intensive, semi-intensive, and extensive. Unlike in Ethiopia, the intensive and semi-intensive dairy farms predominate in Kenya [**Table 1**; (100)], whereas all beef production is by the extensive system. The intensive system is predominant in the Mount Kenya and Central Rift Valley regions, where crop production is also practiced. It is also common in many urban and peri-urban centers in humid and sub-humid areas of the country. In the extensive dairy production system, 3% of farms hold 35% of the dairy cattle population, and 70% of the national livestock herd is found in Kenya's arid and semi-arid lands (100).

In Nigeria there are three dairy cattle production systems: extensive or traditional, semi-intensive (agro-pastoral), and intensive (modern) system [**Table 1**; (101)]. Whereas commercial farms raise imported exotic breeds and their crosses, local breeds predominate. These are mainly managed by semi-sedentary and transhumant pastoralists in large herds. However, compared with herd sizes in North America, these may be considered relatively small; for example, concentrated animal feeding operations (CAFOs) predominate in some countries, with more than 70% of beef produced in the USA in 2002 being derived from CAFOs holding more than 5000 head of cattle (102). Commercially oriented, urban cattle farming has started to emerge in Nigeria, but is still relatively marginal.

As Egypt has limited natural pastures, cattle (and buffalo) production here is well integrated with cropland. There are three main production systems: intensive, semi-intensive, and extensive [**Table 1**; (103)]. The semi-intensive system is dominated by improved local breeds, producing both beef and milk and comprise almost 60% of the total bovine population.

ARE THERE HOT SPOTS FOR ZOONOTIC TRANSMISSION IN AFRICA?

With the majority of articles from Africa indicating the predominance of *C. hominis* in human cases of *Cryptosporidium* infection, and as many livestock infections in Africa are with non-zoonotic species, such as *C. xiaoi*, we tried to identify African studies providing convincing evidence of zoonotic transmission ("black swan" events). The intention was that such studies may indicate risk factors for transmission not occurring in other settings in Africa—given that the overwhelming number of studies suggest that non-zoonotic transmission predominates.

A review of the literature identified some studies in which the titles or abstracts indicated that the authors were considering zoonotic transmission of *Cryptosporidium* in specific settings. Most of these studies were based upon investigating prevalence, sometimes species, and less frequently subtype, of *Cryptosporidium* infections in people and animals residing in similar areas and inferring from these data any potential for zoonotic transmission [e.g., (104, 105)]. Less frequently case-control type studies were reported in which the prevalence and species of *Cryptosporidium* in people reporting close contact with animals were compared with prevalence and species in people reporting little or no contact with animals [e.g., (106)]. However, the large number of possible confounders and difficulties in appropriate matching of those with and without animal contact means the results of such studies are difficult to interpret. Finally, contact-network analysis, in which samples are analyzed from both human and animal contacts of people infected with *Cryptosporidium*, was reported in two papers (48, 76). Those studies that have collected the most information (species, subtype, matched case-control, or contact-network analysis) are likely to provide the strongest indications regarding the likelihood of zoonotic transmission. It is therefore interesting that the two papers providing the most detailed analysis, with molecular investigation of *Cryptosporidium* isolates from humans and animals along with cluster analysis of the results (48, 76), both suggest that animal-to-human transmission is a minor, and probably separate, transmission component, although does occur.

Nevertheless, some studies indicate some likelihood of zoonotic *Cryptosporidium* transmission, and it is thus relevant to consider these more closely. For *C. parvum*, some articles from Egypt, Ethiopia, Kenya, Nigeria, São Tomé and Príncipe, and Tunisia indicate the potential for zoonotic transmission. These are summarized in **Table 2**. We considered each article in

turn, and, based on the information reported, gave a subjective analysis of the strength of evidence (strong, moderate, or weak) of zoonotic transmission provided in the situations described. Thus, articles were only considered where subtype families IIa or IId were identified. The evidence was considered strong if similar subtypes were reported from animals and humans with a plausible connection in time and space, or with other strong epidemiological and statistical evidence of animal contact being a risk factor. The evidence was scored as moderate if the study reported zoonotic species or subtypes from $\geq$ 3 humans, plus at least one of the following: detection by at least one other testing modality (e.g., microscopy), immunocompromised person, and gastrointestinal symptoms. The additional criteria were used to increase the probability that detection reflected infection—either because the symptoms indicated infection or because of host susceptibility. The additional testing modalities were also an attempt to exclude molecular detection reflecting transient passage rather than infection. For articles that presented neither strong nor moderate evidence, the evidence was classified as "weak."

Of the 10 studies identified, only three, two from Egypt, and one from Tunisia, provided strong evidence that zoonotic transmission might have occurred. Thus, on the whole, the majority of articles do not provide convincing evidence of any "hot spots" of zoonotic transmission, and the overall picture is that human infections of *Cryptosporidium* are predominantly *C. hominis* and non-IIa/IId *C. parvum.* Regarding the two countries (Tunisia and Egypt) where the evidence for zoonotic transmission was relatively strong, we speculate cautiously that there are some relevant factors. Both countries are classified as lower-middle income economies and considered to have a relatively strong commercial sector and drive for nationally produced meat and dairy products (113). In addition, due to geographic and climate factors in both countries, cattle-raising land is restricted, being basically only available in the northern areas (specifically the districts of Beja and Bizerte) in Tunisia and along the Nile and in the Nile delta in Egypt. Thus, by necessity, there is close integration between cattle raising (both dairy and beef) and human settlements in both countries. However, with just a few papers from both countries, it is incorrect to label them as transmission hot spots, and many other regions also have close associations between people and cattle raising.

Other articles that indicate zoonotic transmission are concerned with species other than *C. parvum*, with some circumstantial evidence of *C. meleagridis, C. muris, C. canis, C. suis, C. ubiquitum,* and *C. xiaoi* infections in people and associated animals (see **Table 3**). As with the *C. parvum* articles, these too have been evaluated for the strength of evidence indicating zoonotic transmission. Interestingly, we were unable to find any that provided strong evidence of zoonotic transmission, and probably the strongest indicator that these are examples of zoonotic transmission are reflected in that these are typically animal-associated species and that in several of the studies the infected people are immunocompromised, and therefore probably likely to be susceptible to pathogens that do not tend to infect humans. As pointed out in an article that also discusses the public health threat from zoonotic enteric protozoa in wildlife (130), the terminology may be loaded and it is questionable whether a pathogen should be considered zoonotic that usually infects only animals and is reported rarely in low numbers from a highly immunocompromised human patient. The example given in that article is *C. suis*, and such pathogens are described therein as "potentially zoonotic" (130).

THE PREDOMINANCE OF ANTHROPONOTIC TRANSMISSION OF *CRYPTOSPORIDIUM* IN AFRICA: A REVIEW OF THE EVIDENCE

Summary of Why Zoonotic Transmission May Be Expected in Africa

Based on the preceding sections of this article, as well as on previous reviews on *Cryptosporidium* infections in Africa (51, 52), it is clear that transmission of *Cryptosporidium* infection of people in Africa is currently largely anthroponotic (human to human), being mostly *C. hominis* or non-zoonotic *C. parvum*. However, from a superficial perspective, there are several factors that would suggest that zoonotic transmission in Africa would be at least as likely, if not more likely, to occur than in some other parts of the world. First, the relationships between animals and their owners in Africa are often much closer than in, for example, European countries; in Africa, people and their livestock may literally share the same sleeping quarters. Secondly, there is probably a greater likelihood of contamination of drinking water supplies by livestock in Africa than in many parts of the world, with water supplies often limited, and a general absence of catchment control for protection of water supplies, with surface waters used both as drinking water supplies and also for watering animals (82, 87, 131, 132). Thirdly, animals are often closely associated with the growth of fruit and vegetables that are consumed raw—with animals being involved in the plowing, harvesting, and transport of such crops, and often standing close by in marketplaces where such crops are sold (132). Fourthly, in many parts of Africa, there is close contact between people and animal manure, which is used as a resource for fertilizer, fuel, and building materials, with the pats often prepared by hand, frequently by women (also frequently involved in food preparation) and young children (99, 133). Indeed, animals in the domestic environment have been cited as being a contributor to the substantial burden of zoonotic disease, including cryptosporidiosis (134), either directly or indirectly (132). Although Aldeyarbi et al. (51) comment that animals are not found in close proximity to residences in urban settings in Africa, this seems not to be the case. For example, a survey from Burkina Faso indicated that more 25% of households in Ouagadougou kept livestock (135) and in 2019, FAO noted the potential zoonotic dangers associated with livestock in rapidly expanding African cities (136).

Thus, with this apparently great potential for zoonotic transmission in African countries, the question arises about why it does not seem to occur to a greater extent. We put forward the following possible reasons, and suggest that all or some of these may play a role. We also suggest that the large number

TABLE 2 | Overview of articles for which zoonotic transmission of *C. parvum** in Africa is suggested.

Country	Evidence of zoonotic transmission provided	Evaluation of evidence**	References
Egypt	Identical subtypes of *C. parvum* IIa and IId found in stool from cattle, buffalo and in 7 children (with diarrhea), from the same area	Strong	(105)
Egypt	Identical subtypes of *C. parvum* IId found in cattle, buffalo and in 5 humans, in the same area; age and symptoms not reported	Strong	(107)
Egypt	*C. parvum* IIa and IId in 2 children with diarrhea in a childcare center; animal contact reported	Weak/moderate	(68)
Ethiopia	*C. parvum* IIa in 9 adults (5 HIV positive) and 3 HIV negative children, with diarrhea, microscopy positive; various regions; sampling strategy unclear	Moderate	(108)
Ethiopia	*C. parvum* IIa and IId in 71 and 5 adult HIV patients, respectively, associated with diarrhea and contact with calves	Moderate/strong	(65)
Kenya	*C. parvum* IIa in 5 adult HIV patients; 3 with and 2 without diarrhea	Moderate	(109)
Nigeria	*C. parvum* IIa in 2 healthy children; microscopy positive	Weak	(64)
São Tomé and Príncipe	*C. parvum* IIa and IId in 2 and 3 pediatric hospital patients, respectively, microscopy positive; symptoms not specified	Moderate	(110)
Tunisia	Identical subtypes of *C. parvum* IIa and IId found in stool from calves and 4 children (3 with diarrhea), from the same area	Strong	(111)
Tunisia	*C. parvum* IIa found in 8 adult patients and IId found in 4 pediatric and 5 adult patients; symptoms not specified	Moderate	(112)

**Only C. parvum gp60 allele families IIa or IId considered in this table (IIc, IIe and IIm gp60 allele types are likely anthroponotic and the evidence for other allele families being zoonotic is inconclusive).*

***Strong: same zoonotic species or subtypes detected in ≥ 1 humans and animals with a plausible connection in time and space, or with other strong epidemiological and statistical evidence of animal contact being a risk factor.*

Moderate: detection of zoonotic species or subtype in ≥3 humans, plus at least one of the following: gastrointestinal symptoms, immunocompromised, detection by ≥1 other testing modality.

Weak: other detection of zoonotic subtype.

of articles from Africa apparently suggesting the importance of zoonotic transmission in these settings, may be perpetuating a misleading myth.

Are *C. parvum* Infections Relatively Infrequent in African Livestock?

Our first suggestion is that, *C. parvum* infection is not well established among the ruminant livestock populations in many regions in Africa. Although larger-scale herds do occur in some parts of Africa, as detailed in the section of this article on livestock production, livestock rearing is usually extensive, pastoral, or semi-pastoral. Although there are notable exceptions, and some large cattle enterprises may be found, in general the average number of cattle per farm is around 50. A multivariable analysis of risk factors for pre-weaned calves acquiring *C. parvum* infection and *C. bovis* infection has demonstrated an increased risk of *C. parvum* infection with greater herd size, with calves in herds of over 200 animals being at significantly greater risk of infection than calves in herds of below 100 animals (137). Furthermore, with high mortality of neonates and year-round, relatively low production rates, the neonatal density in African herds tends to remain low and constant; peaks in zoonotic transmission at the same time as seasonal lambing and calving are well recognized in some non-African countries (22, 138).

In addition, other relevant factors that significantly increased the risk of *C. parvum* infection in calves was mean monthly precipitation of 100–150 mm (compared with below 100 mm), being housed inside, and the use of hay bedding (137). These factors are thus those that favor close contact between animals (herd size and housing) and oocyst survival (hay bedding and precipitation); the association with hay bedding has also been reported from a study in Mexico (139). These risk factors for *C. parvum* infection in calves are therefore often lacking in the cattle-husbandry systems predominant in many African countries, with most herds being below 100 animals. Furthermore, even in places where large herd sizes may occur (e.g., in Burkina Faso), these are often being managed in pastoral systems where other risk factors (e.g., housing and hay bedding) are lacking (140). Furthermore, in such animal management systems, exposure to the climate is also likely to be detrimental to transmission, with desiccation and UV exposure also playing a part (see the later section on oocyst survival in the African envirnoment). Indeed, a study from Tanzania (47) in which a notable lack of *Cryptosporidium* infection was identified among 601 dairy calves from different management systems, small herd size and climatic factors were considered to be important factors that could have reduced the potential for establishment of infection and/or contributed to disease fade out (141). Similar arguments may also be proposed for why *C. parvum* may be less established in small ruminants in various African countries (142).

Another potential factor of importance for the lack of infection with *C. parvum* in African cattle is cattle breed (and, correspondingly, could also be relevant for small ruminant livestock). As noted by Chang'a et al. (47), most studies on *Cryptosporidium* in cattle involve *Bos taurus* breeds, but *B. indicus* breeds (which often predominate in African farms) may

TABLE 3 | Overview of articles for which zoonotic transmission of *Cryptosporidium* species other than *C. parvum* in Africa is suggested.

Country	Evidence of zoonotic transmission provided	Evaluation of evidence*	References
C. meleagridis			
Côte d'Ivoire	9 people with intestinal disorders attending village primary healthcare centers; *C. meleagridis* also found in 4 chickens; age or symptoms not further specified	Moderate	(57)
Egypt	Patients with gastrointestinal symptoms ($n = 2$)	Weak	(114)
Equatorial Guinea	HIV infected female, also positive by antigen test and microscopy; symptoms not specified	Weak	(115)
Ethiopia	3 HIV positive children; symptoms not specified	Moderate	(65)
Gabon	1 child with diarrhea; but no confirmed transmission cluster involving animal contacts (however, birds not sampled)	Weak	(76)
Ghana	3 children with diarrhea; but no confirmed transmission cluster involving animal contacts (however, birds not sampled)	Moderate	(76)
Kenya	HIV positive adult patients, 2 with diarrhea, 1 without diarrhea	Moderate	(108)
Kenya	6 pediatric patients; microscopy positive; symptoms not specified	Moderate	(116)
Kenya	1 HIV infected patient; microscopy also positive, age or symptoms not specified	Weak	(117)
Kenya	1 person, no demographic or clinical information	Weak	(118)
Kenya	1 HIV positive adult; symptoms not specified	Weak	(119)
Kenya	1 child presenting to hospital; microscopy positive; symptoms not specified	Weak	(120)
Kenya	2 pediatric patients with diarrhea; microscopy positive	Weak	(59)
Kenya	1 child with diarrhea; ELISA antigen also positive	Weak	(121)
Madagascar	5 children with diarrhea and 2 neighboring children of children with *Cryptosporidium* diarrhea; but no confirmed transmission cluster involving animal contacts (however, birds not sampled)	Moderate	(76)
Malawi	2 pediatric patients with diarrhea; microscopy positive; rural area	Weak	(122)
Nigeria	5 asymptomatic children; microscopy positive	Moderate	(64)
Nigeria	HIV-positive adult, asymptomatic; *C. meleagridis* also detected in 1 chicken from same area	Weak	(58)
South Africa	1 child hospitalized with diarrhea; gp60 subtype was IIId (found in humans in India, but not reported in animals)	Weak	(123)
South Africa	1 child from a clinic; microscopy positive; symptoms not specified	Weak	(66)
Tanzania	1 child with diarrhea and 1 neighboring child with *Cryptosporidium* diarrhea; but no confirmed transmission cluster involving animal contacts	Weak	(76)
Tunisia	3 children without diarrhea; microscopy positive	Moderate	(110)
Tunisia	2 adult HIV patients, both with gp60 subtype IIIbA26G1R1; 1 immunocompromised child, not subtyped, in co-infection with *C. hominis*; symptoms not specified	Moderate	(111)
Tunisia	2 children with primary immunodeficiency and diarrhea, one a co-detection with *C. hominis*; microscopy positive	Weak	(124)
Uganda	3 hospital admitted children with persistent diarrhea	Moderate	(125)
C. muris			
Kenya	1 HIV positive adult with diarrhea; microscopy positive	Weak	(126)
Kenya	1 child presenting to hospital; microscopy positive; symptoms not specified	Weak	(120)
Malawi	1 child with diarrhea; co-detection with *C. andersoni*	Weak	(122)
Nigeria	1 child with diarrhea; *C. muris* also detected in 1 goat in same area	Weak/moderate	(58)
C. felis			
Ethiopia	1 HIV positive child; symptoms not specified	Weak	(65)
Gabon	1 household contact of a child with *Cryptosporidium* diarrhea	Weak	(76)
Ghana	2 children with diarrhea; 1 household contact of child with *Cryptosporidium* diarrhea; 3 neighboring children of children with *Cryptosporidium* diarrhea; but no confirmed transmission cluster involving animal contacts	Moderate	(76)
Kenya	4 pediatric patients with diarrhea; microscopy positive	Moderate	(59)
Kenya	2 children presenting to hospital; microscopy positive; symptoms not specified	Weak	(120)
Nigeria	2 adult HIV patients; symptoms not specified	Weak	(127)
Nigeria	1 adult HIV patient; symptoms not specified	Weak	(128)
Tanzania	2 neighboring children of children with *Cryptosporidium* diarrhea; but no confirmed transmission cluster involving animal contacts; symptoms not specified	Weak	(76)

(Continued)

TABLE 3 | Continued

Country	Evidence of zoonotic transmission provided	Evaluation of evidence*	References
C. canis			
Ethiopia	2 HIV positive children; symptoms not specified	Weak	(65)
Kenya	3 children presenting to hospital; microscopy positive; symptoms not specified	Moderate	(120)
Kenya	HIV positive adult patients, 2 with diarrhea, 2 without diarrhea	Moderate	(109)
Kenya	2 pediatric patients; microscopy positive; symptoms not specified	Weak	(116)
Kenya	1 child with diarrhea; ELISA antigen positive	Weak	(121)
Nigeria	1 adult HIV patient; symptoms not specified	Weak	(127)
Nigeria	1 asymptomatic child; microscopy positive	Weak	(64)
C. suis			
Kenya	HIV positive adult patients, 1 with diarrhea, 1 without diarrhea	Weak	(109)
Madagascar	1 adult, no diarrhea; *C. suis* detected in 3 pigs in the same village	Weak	(129)
Madagascar	1 child neighbor of a child with *Cryptosporidium* diarrhea; symptoms not specified; no confirmed transmission cluster involving animal contacts	Weak	(76)
C. ubiquitum			
Nigeria	3 asymptomatic children; microscopy positive (reported as "*Cryptosporidium cervine* genotype")	Moderate	(64)
C. cuniculus			
Nigeria	5 asymptomatic children; microscopy positive (reported as "*Cryptosporidium rabbit genotype*")	Moderate	(64)
C. xiaoi			
Ethiopia	2 HIV positive children; symptoms not specified	Weak	(65)
Ghana	1 child with diarrhea (*C. xiaoi*/*bovis);* also identified in 19 goats and 5 sheep in the same region	Weak/moderate	(76)

**Strong: same zoonotic species or subtypes detected in ≥ 1 humans and animals with a plausible connection in time and space, or with other strong epidemiological and statistical evidence of animal contact being a risk factor.*
Moderate: detection of zoonotic species or subtype in ≥ 3 humans, plus at least one of the following: gastrointestinal symptoms, immunocompromised, detection by ≥ 1 other testing modality.
Weak: other detection of zoonotic species or subtype.

be more resistant. A study from Nigeria (143) involving 195 calves of the White Fulani and Sokoto Gudali breeds (both *B. indicus* breeds) reported 16% prevalence of *Cryptosporidium*, but none were *C. parvum*. Although systematic investigation of *Cryptosporidium* infections in general, and *C. parvum* in particular, are lacking, different management routines may be associated with different breeds [e.g., some breeds of cattle, particularly European taurine breeds, tend to need to be housed indoors due to their susceptibility to African trypanosomiasis; (144)]. A study from Malaysia (145) also supports the suggestion that particular breeds may be more susceptible to *Cryptosporidium* infection, with significantly higher prevalences of infection reported from Mafriwal cattle (Sahiwal × Friesian crosses) and from Jersey × Friesian crosses.

In Africa, a study from Zambia (70) considered that the significantly higher prevalence of *Cryptosporidium* infection in dairy-farm cattle compared with beef calves or "traditionally reared" calves was due to the management factors. These include higher density of dairy calves favoring propagation of infection in confined housing, whereas calves in extensively reared beef and traditional husbandry systems were not only fewer in number but outside, where any oocysts would be exposed to environmental pressures such as desiccation and direct sunlight, resulting in a reduced infection pressure (70). Cattle breed may also have played a role, as the calves on the dairy farms were Jersey, Friesian, or crossbreeds, but a mixture of cattle breeds predominated in the other management systems, including Brahman and Boran, both *B. indicus* breeds. Similarly, based on the results of their study in two relatively large dairy farms in the central highlands of Ethiopia (49), exotic breeds (Holstein-Friesian; *B. taurus*) were suggested as being more vulnerable to *Cryptosporidium* infection than the local Zebu breed (*B. indicus*), as crossbred calves with a greater proportion of Holstein-Friesian "blood" had a higher prevalence of *Cryptosporidium* infection than calves with a lower proportion. Considering the three articles that we considered showed relatively strong evidence of zoonotic transmission of *C. parvum*, only the one from Egypt (105) mentions breed; however, although the authors state that, in general, most livestock in the region of the study were native crossbreeds, the animal breeds in the study, or in the two specific farms where zoonotic transmission was suggested, were not stated.

Thus, although disentangling the potential risk factors from each other is clearly difficult, it is also apparent that for various reasons, under current conditions, African cattle may be generally less likely to be shedding *C. parvum* oocysts than cattle elsewhere. However, this does not exclude this parasite establishing and spreading in African livestock populations as

circumstances change. Such a scenario could be devastating for African livestock production, as well as having potential for disseminating further to people.

Could People in Africa Be Less Susceptible to Zoonotic *Cryptosporidium* Infection?

This leads us to explore whether the "other part" of a potential zoonotic transmission cycle may also exhibit some factors that contribute to the relative lack of this transmission route; namely the potential human hosts. Although animal-human contact is probably more extensive in Africa than in other areas of the world, there may be some aspects of people in Africa that result in them having a different probability of acquiring zoonotic *Cryptosporidium* infection than elsewhere. Of particular relevance in this respect is the relatively high prevalence of infection with *C. hominis* in young children in many African countries (5, 25–27); babies and young infants are generally more likely to be exposed to *Cryptosporidium* oocysts from human infections than from animal infections. Thus, it seems likely that *C. hominis* infections early in life may provide some protection against infection with zoonotic *Cryptosporidium* species later. Although immune responses to cryptosporidiosis are currently not completely understood, it is clear that both innate and adaptive immune responses have a relevant role in both protection from, and resolution of, *Cryptosporidium* infections and cryptosporidiosis. However, the level of immunity has not been determined, nor the extent to which there is cross-protection between different species of *Cryptosporidium*. An early human experimental study with *C. parvum*, in which primary infection of seronegative adults with a challenge dose was followed by another challenge with the same *C. parvum* oocyst isolate approximately 1 year later, showed that initial exposure may be insufficient to protect against clinical illness 1 year later (146). However, it is likely that young children receive several low-level exposures, and this may have a different outcome to that described in the human-challenge study. In a later study, adults with pre-existing anti-*C. parvum* serum IgG only became infected when challenged with higher *Cryptosporidium* oocyst doses, and did not excrete as many oocysts, indicating that prior exposure to *C. parvum* does provide protection from infection and disease at lower oocyst doses (147). Using another approach to investigate exposure protection, serological investigations in two UK cities with high and low incidences of reported cryptosporidiosis indicated that exposure to non-pathogenic strains of *Cryptosporidium* or repeated low-level exposure to pathogenic strains could provide a protective effect (148). Indeed, it has been suggested that by eliminating a source of low-level *Cryptosporidium* oocyst exposure may, paradoxically, increase the risk of symptomatic infection from other exposure sources (149).

Regarding evidence of whether exposure to *C. hominis* provides cross-immunity against subsequent *C. parvum* challenge, analysis of antibody responses in children in Peru (150) and Bangladesh (151) suggests that *C. hominis* infection results in development of notable antibody responses against *C. parvum* antigens, indicating that these responses are directed toward epitopes conserved across species and subtypes. However, the importance of this is unclear as the roles of B-cells and antibody responses in cryptosporidiosis remain controversial, although they do seem to contribute to protection (152). T-cell mediated responses, particularly with CD4+ type-1-cells, are, recognized as a more crucial component. An experimental study using gnotobiotic piglets found that a substantial (one million oocysts) *C. hominis* challenge conferred full immunity against further challenge with the same *C. hominis* isolate, and partial immunity (i.e., infected but with significantly lower oocyst excretion than non-challenged controls) when further challenged by a substantial (10 million oocysts) *C. parvum* oocyst dose (153). Again, these large dose experiments beg the question regarding whether trickle exposures may be similarly (or more) likely to provide protection against future challenge, including with *C. parvum* or other species.

However, although exposure to *Cryptosporidium* early in life may be relevant for limiting future infection (as older children with responsibility, for example, for herding small ruminants), including with zoonotic species, other risk factors may occur in Africa that may increase infection likelihood. These could include concurrent infections or conditions that may limit the robustness or effect of an immune response against future challenge. It is well known that most people living with HIV reside in Africa, with 25.7 million estimated by World Health Organization in 2018, compared with 3.8 million in southeast Asia and 3.5 million in the Americas (154). Cryptosporidiosis is known as one of the major causes of diarrhea in patients with HIV, and is associated with significant morbidity and mortality in the AIDS population; a recent systematic review and meta-analysis indicated that the pooled prevalence of *Cryptosporidium* in HIV-positive patients in Africa was around 11.9% (CI: 8.8%−16.0%) (155), only marginally below that of SE Asia, which topped the list at 12.7% (CI: 9.7%−16.4%). It is not surprising that reports on zoonotic *Cryptosporidium* infections in Africa with species other than *C. parvum* are often in people with immunodeficiencies, particularly from HIV. Of the 51 articles listed in **Table 2**, 17 refer to infections in people with immunodeficiencies. It is also clear that other insults to human health, including malnutrition and other infections that may occur more commonly in some African countries, may not only exacerbate symptoms but also contribute toward individuals being more susceptible to infection due, among other reasons, to suppressed immunity.

In addition to immune effects of infections and other factors, genetic variations in the population itself may make individuals, or populations, more or less susceptible to specific infections. For cryptosporidiosis, candidate gene studies indicating an increased risk of cryptosporidiosis include HLA class I and II genes, SNPs in the mannose binding lectin (MBL) gene, and variation within the protein kinase C alpha (PRKCA) gene (156). Some of these variations may be of particular relevance to Africans; for example, it has been noted that the median MBL protein concentration in blood is considerably lower in Africans than in other racial groups (157), whereas, in contrast, the "risk" T allele in the PRKCA gene is reported to occur at relatively low frequencies in Africa, and least frequently in West Africa (156). Whether these

genetic variations may affect zoonotic transmission (zoonotic *C. parvum* infection) has not yet been explored.

Is the African Environment More Detrimental to Oocyst Survival?

Finally, the third player in zoonotic transmission of *Cryptosporidium*, is the environment. *Cryptosporidium* oocysts have long been recognized as being robust to many environmental pressures (158), and is one reason why waterborne and foodborne transmission occurs. Some African environments, with low humidity and high UV index, may have a negative impact on oocyst survival. Although not all of Africa is continuously dry and sunny, and there are extremes of weather and temperature, in general, in several places in Africa where livestock are grazed, environmental conditions may not be optimal for prolonged oocyst survival. This reduces the likelihood of animal-to-animal transmission (see previous section), and also animal-to-human transmission. Whether different species or subtypes of *Cryptosporidium* oocysts may have greater environmental robustness has been scarcely investigated, but a tentatively forwarded hypothesis (63) is that mutation in the COWP9 gene, which as other genes in the COWP family are associated with oocyst wall formation (159) may affect robustness, and thus transmission possibilities.

Where water is contaminated with feces of an infected individual, the potential for oocyst survival, and thus onward transmission, increases. As animals may have greater opportunities to contaminate drinking water in Africa than in other parts of the world, this may argue for an increased likelihood of zoonotic transmission of *Cryptosporidium* in Africa rather than in places where catchment protection measures are the norm. Indeed, Vermeulen et al. (160) note that the *Cryptosporidium* load from manure could be reduced substantially in several African countries by manure treatment with elevated temperatures, such as composting. Nevertheless, data modeling indicates that human, rather than animal, feces are the more predominant source of oocyst contamination, with most contamination around growing urban centers, and with the potential of these urban hot spots to grow and multiply as sewer connections are installed without corresponding and appropriate sewage treatment (89).

Contamination of water sources with *Cryptosporidium* oocysts varies over shorter timesteps than say, monthly averages, with water contamination likely to respond strongly to major weather events, such as prolonged and heavy rain or flooding events. It should be noted that oocysts of animal origin in runoff from grazing land are more likely to have been already inactivated, than oocysts from overloaded human sewerage systems that have spent less time exposed to desiccation and UV radiation. Of potential relevance in this context, is that even in some countries where *C. parvum* tends to be the predominant species associated with sporadic human infections, *C. hominis* seems to be the most usual species associated with waterborne outbreaks. One example is Sweden, where *C. parvum* causes most sporadic cases of cryptosporidiosis [between 2006 and 2008, there was just under double the number of *C. parvum* cases compared with *C. hominis* cases, with most *C. hominis* cases infected abroad; (161)], but major waterborne outbreaks in this country have been associated with *C. hominis* [e.g., (162)]. The subtype here was IbA10G2, which is discussed in greater detail in the section on species and genotypes. That this subtype occurs rarely in Africa but is associated with large-scale outbreaks elsewhere, may suggest that African populations are at risk should it be introduced; alternatively, should infection with this subtype be particularly associated with the T allele in the PRKCA gene (156), then African populations may be partially protected.

CONCLUSIONS

That both zoonotic and anthroponotic transmission of *Cryptosporidium* occur has long been accepted, and it is also well established that these routes are associated with different *Cryptosporidium* species and subtypes. However, in-depth exploration of transmission patterns and what they mean for public and veterinary health and interventions is scanty. In our opinion, it is all too common for publications to quote the serious toll that cryptosporidiosis takes on pediatric health in countries in Africa (and other low-income areas) and tie those figures to the potential for zoonotic transmission of this parasite and the necessity of a One Health perspective [e.g., (163)]. While we applaud the One Health approach, such juxtapositions can be misleading for some readers. Establishment of the facilities for more identification of species and subtyping of *Cryptosporidium* in different African countries in the coming years will provide further data regarding relative occurrences in different countries, hosts, and situations, and will be an essential tool for implementing appropriate control measures. This calls for not only more sophisticated laboratory infrastructure, but also scientists trained in the various techniques and with the appropriate skillsets and knowledge for such investigations.

It is essential that we are aware that in much of Africa, and probably for a variety of reasons, as discussed in preceding sections, anthroponotic transmission predominates at present. We add the words "at present" with emphasis; we want to stress that the current situation can change and is probably changing. Globalization may result in introduction of new species/subtypes of *Cryptosporidium*; it is common to think that visitors to Africa from Europe, for example, may return home with diarrheal pathogens. But it is of equal or greater importance to think that they may also export specific currently non-established *Cryptosporidium* subtypes *to* Africa, including *C. hominis* IbA10G2 and *C. parvum* IIaA15G2R1. Both of these are hypothesized to be hypertransmissible (55), but apparently occur only rarely in Africa currently; if these specific subtypes are globally hypertransmissible, rather than merely well suited and established in their current niches, then their introduction to areas of Africa could be disastrous for both human and animal health. In addition, changes in farm management (e.g., less extensive farming, more intensive farming, more urban farms) may result in animal feces being less exposed to environmental pressures that inactivate *Cryptosporidium*

oocysts and also increase the possibilities of between-cow transmission, and hence environmental contamination and infection pressure.

It may be of relevance that the two countries that seemed to have clearest evidence of zoonotic transmission of *C. parvum* (Tunisia and Egypt), and from where subtype IIaA15G2R1 has been reported, have relatively limited regions suitable for livestock rearing and thus the potential for direct or indirect (water contamination) transmission may be exacerbated by the requirement for high animal densities in a restricted area.

Although reducing pediatric cryptosporidiosis in Africa, with its substantial mortality and morbidity burden, should clearly be a goal, it should also be borne in mind that reduced childhood immunity may, at population level, result in an epidemiological shift from an "endemic and predominantly anthroponotic" toward an "epidemic and predominantly zoonotic" pattern. Obviously, the negative impact of diarrheal disease is more damaging in young children, but slightly older children with acute malnutrition, or children or adults with untreated HIV, are also vulnerable groups. Although the extent of waterborne transmission of cryptosporidiosis in Africa is almost impossible to determine, a water, sanitation, and hygiene (WASH) perspective is a fundamental concept to limit the transmission of any diarrheal pathogen, zoonotic or not; however, WASH initiatives should be "transformative" in order to have a lasting and substantial impact (164). At the other end of the technology scale, as genome sequencing studies uncover relevant mutations, we will gradually gain greater information that may provide the basis for implementation of different approaches to limit, or prevent, both anthroponotic and zoonotic transmission.

AUTHOR CONTRIBUTIONS

The theme of this article was proposed by LR and derived from discussion with all co-authors. AE had main responsibility for section Human Health Impacts, TK and GT for sections Veterinary Health Impacts and Ruminant Livestock Production in Africa and Potential Effects on *Cryptosporidium* Transmission, ØJ for sections *Cryptosporidium* Infections in Africa: Distributions of Species/Genotypes and Are There Hot Spots for Zoonotic Transmission in Africa? and **Tables 2**, **3**. LR had main responsibility for all other sections, and for overall structure. All authors commented on all sections of the drafts and approved the final submission.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.575881/full#supplementary-material

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Diversity of *Eimeria* Species in Wild Chamois *Rupicapra* spp.: A Statistical Approach in Morphological Taxonomy

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Wildlife is frequently infected by intestinal protozoa, which may threaten their fitness and health. A diverse community of *Eimeria* species is known to occur in the digestive tract of mountain-dwelling ungulates, including chamois (genus *Rupicapra*). However, available data on *Eimeria* diversity in these taxa is at times inconsistent and mostly dated. In the present study, we aimed to revisit the occurrence of *Eimeria* spp. in the Alpine subspecies of the Northern chamois (*Rupicapra rupicapra rupicapra*) and the Apennine subspecies of the Southern chamois (*Rupicapra pyrenaica ornata*) in Italy, using an integrated approach based on a hierarchical cluster analysis (HCPC) applied to oocyst morphology and morphometry. A total of 352 fecal samples were collected from *R. r. rupicapra* ($n = 262$) and *R. p. ornata* ($n = 90$). Overall, 85.3% (300/352) of the animals tested microscopically positive to *Eimeria* spp. Based on morphological analysis, we identified all the eimerian species described in chamois. Through the HCPC method, five clusters were generated, corresponding to *E. suppereri, E. yakimoffmatschoulskyi, E. riedmuelleri* (two different clusters), and *E. rupicaprae* morphotypes. The well-defined clusters within *E. riedmuelleri* support the existence of two distinct morphological groups, possibly referable to different taxonomic units. This study suggests that combining a morphometrical approach with a powerful statistical method may be helpful to disentangle uncertainties in the morphology of *Eimeria* oocysts and to address taxonomic studies of eimeriid protozoa at a specific host taxon level.

Keywords: *Eimeria* spp., *Rupicapra* spp., morphology, hierarchical clustering, Italy

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INTRODUCTION

Exploring the parasite communities in wild animals represents a main challenge for wildlife management, as several parasites may have an impact on their fitness and health, even more so in the frequent event of co-infections (1). Moreover, wildlife can play an important role as reservoirs of pathogens of medical and/or veterinary importance (2).

Wild caprines (Bovidae, Caprinae) are reportedly known to harbor rich parasite communities including representatives of the genus *Eimeria* Schneider, 1875 (Coccidia, Apicomplexa) (3). In particular, five *Eimeria* species have been described to infect the iconic members of the genus *Rupicapra*: *Eimeria alpina* Supperer and Kutzer, 1961; *Eimeria riedmuelleri* Yakimoff and Matschoulsky, 1940; *Eimeria rupicaprae* Galli-Valerio, 1924; *Eimeria suppereri* Kutzer, 1964 and *Eimeria yakimoffmatschoulskyi* Supperer and Kutzer, 1961 (4). These species have never been reported in other ruminants, thus advocating for their strict host specificity.

Traditionally, *Eimeria* species identification is obtained using a set of biological traits and morphological features such as the identity of the host species as well as oocyst size, shape and structure (curvature, presence/absence of oocyst residuum, conspicuous/inconspicuous micropyle), shape and structure of the sporocysts (5, 6). However, morphological methods are often challenging and several other *Eimeria* species from *Rupicapra* spp. have been inadequately or erroneously described. As stated in Levine and Ivens (4), the name *Eimeria longispora* Rudovsky 1922, identified in chamois from Austria, should be considered as *nomen nudum*, due to its incomplete description; moreover, the report of *Eimeria arloingi*, *Eimeria crandallis*, *Eimeria ninakohlyakimovae*, and *Eimeria parva* in *R. rupicapra* in the present-day Slovakia and of *E. arloingi* and *E. ninakohlyakimovae* in the present-day Slovenia (7, 8) should be considered uncertain; finally the occurrence of *Eimeria faurei* in hosts other than *Ovis* and *Capra* is doubtful.

In Italy, two taxa of chamois are present (9): the Alpine subspecies of the Northern chamois (*Rupicapra rupicapra rupicapra*), widely spread along the Alps, and the Apennine subspecies of the Southern chamois (*Rupicapra pyrenaica ornata*), which occurs in five protected areas of central Apennines. Due to its limited and fragmented distribution range as well as the small population size, *R. p. ornata* is currently included in the International Union for Conservation of Nature (IUCN) red list in the category of "vulnerable" taxa (VU D1+2) (www.iucn.it). Despite the relevance of *R. r. rupicapra* and *R. p. ornata,* which cover a large geographical area, data on *Eimeria* spp. in wild chamois from Italy are not only limited but also still leave open questions on their identity and prevalence. Indeed, in the Alpine chamois *R. r. rupicapra*, *E. rupicaprae* identification dates back to 1950's through a parasitological survey on the fauna of the Gran Paradiso National Park (Western Alps) (10) and about 20 years later, *E. rupicaprae*, *E. riedmuelleri,* and *E. yakimoffmatschoulskyi* were described in Eastern Alps (11). More recently, Stancampiano et al. (12) confirmed the presence of *E. riedmuelleri* and *E. yakimoffmatschoulskyi* in *R. r. rupicapra*, and recorded *E. suppereri* for the first time in Italy; more intriguingly, a coccidian species resembling *E. faurei*, a species related to domestic sheep was also described (12). As regards the Apennine chamois *R. p. ornata*, only one survey has been carried out in Italy, reporting *E. rupicaprae* and *E. riedmuelleri* (13). Furthermore, *E. alpina* and *E. yakimoffmatschoulskyi* have been recorded by Rossi et al. (14).

Despite the recent advances in morphological, morphometrical, statistical, and molecular biology-based approaches, which may be utilized to investigate the identity of coccidian oocysts, the use of a single methodology is unable to fully characterize these structures and different tools should be applied for taxonomic purposes (6). Therefore, in order to overcome the issues related to the traditional approach to taxonomy of *Eimeria*, the aim of this work was to combine morphological characterization of the oocysts with a statistical method to refine knowledge of *Eimeria* species in Alpine and Apennine chamois.

MATERIALS AND METHODS

From September 2013 to November 2015, fecal samples (n = 262) were collected from *R. r. rupicapra* in Italian Central Alps. The chamois originated from (i) a hunting territory in Lombardy region, with an area of 253 km^2 (45°59'N, 9°32'E) (A); (ii) two contiguous areas in northern Piedmont (46°07'N, 8°17'E) with different population management: a hunting district (B), which extends over 727 km^2 and a protected area (C) of 85, 39 km^2 where hunting is banned (**Figure 1**), and fresh stool samples were collected from the ground soon after defecation. In the same period, fresh fecal samples of *R. p. ornata* (n = 90) were collected after observing defecation, from individuals grazing on upper grasslands, in three subareas of the Abruzzo, Lazio and Molise National Park (D to F, **Figure 1**). All samples were stored in 2.5% potassium dichromate in a 50-ml tight screw cap plastic tube under constant aeration for sporulation for a minimum of two weeks at room temperature until microscopical analysis.

Eimeria oocysts were recovered by flotation in saline solution (density 1,200), while quantitative analysis was performed using a McMaster technique, with a lower detection limit of 50 oocysts per gram of feces (o.p.g.) (15). Morphometrical and morphological features of oocysts and of sporocysts were used for species identification based on the description in Levine and Ivens (4). In particular, their shape, width and length, the oocyst color, rough or smooth wall, the presence/absence of the oocyst micropyle and/or cap, were carefully scrutinized and photographed using a Leica DMD108 microscope, equipped with an integrated camera and image analysis system, by 60 X objective lenses. All measurements are in micrometers (μm).

A statistical analysis with R software v 1.1.463 (FactoMine and factoextra packages) was performed to obtained data, using the Hierarchical Clustering on Principal Components (HCPC) approach on a dataset of 292 sporulated oocysts. The parameters used were length, width and the length/width ratio (Shape index, L × W) for both oocysts and sporocysts. We decided to employ the HCPC approach as it makes possible to combine the three standard methods used in multivariate data analyses (16): (i) principal component methods (PCA, CA, MCA, FAMD, MFA), (ii) hierarchical clustering, and (iii) partitioning clustering, particularly the k-means method. Furthermore, the HCPC analysis allows the characterization of clusters of specimens based on all characters and on subsets of characters, weighting all characters equally.

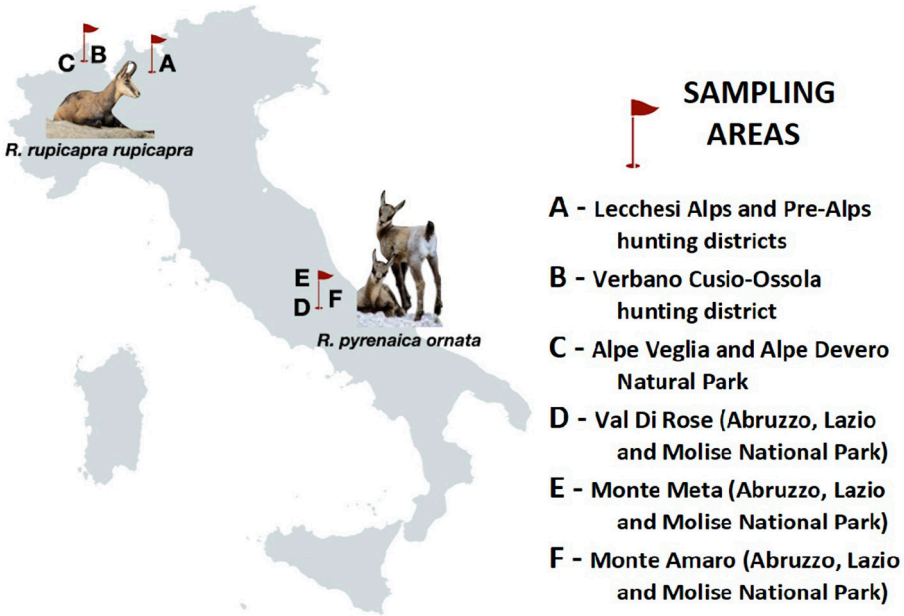

FIGURE 1 | Schematic map of Italy showing the geographical location of sampling sites (A–F) and animal hosts analyzed in the present study.

TABLE 1 | Morphological data of *Eimeria* oocysts and sporocysts isolated from Italian chamois (*Rupicapra* spp.).

Species identification (Cluster)	Oocysts			Sporocysts			Host
	Length Mean Min-max	Width Mean Min-max	Shape index	Length Mean Min-max	Width Mean Min-max	Shape index	
E. suppereri (Cluster 1)	47.54 46-49.08	35.38 34.5-36.26	1.34	19.48 17.5-21.47	10.88 10-11.76	1.79	*R. r. rupicapra*
E. yakimoffmatschoulskyi (Cluster 2)	29.71 25.25-35.97	21.3 18.14-25.64	1.39	13.74 8.9-17.01	6.92 5.2-8.66	1.98	*R. r. rupicapra* *R. p. ornata*
E. rupicaprae (Cluster 3)	27.02 22.24-32.64	22.33 18.5-27.50	1.21	11.75 7.01-17.4	7.71 5.9-11.13	1.5	*R. r. rupicapra* *R. p. ornata*
E. riedmuelleri Spherical form (Cluster 4)	18.28 14.63-22	16 14.25-20.78	1.13	7.57 5.93-9.66	5.99 4.67-7.18	1.26	*R. r. rupicapra* *R. p. ornata*
E. riedmuelleri Ovoid/ellipsoidal form (Cluster 5)	20.21 15.3-24.42	17.4 14.16-21.52	1.16	8.14 6.31-10.45	6.24 4.59-8.35	1.3	*R. r. rupicapra* *R. p. ornata*
E. alpina (-)	11.12 10.4-11.85	11 10.15-11.85	1	-	-	-	*R. r. rupicapra*

Arithmetic mean of measurements of length, width and shape index, with minimum and maximum values are indicated for all Eimeria species analyzed in the present study. All measurements are in micrometers.

RESULTS

Overall, 85.3% (300/352) (95%, C.I. = 81.5–89.1) of samples were microscopically positive to *Eimeria* spp., with a mean intensity of up to 776 o.p.g. Prevalence in *R. r. rupicapra* was 81.2% (213/262) (95%, C.I. = 77.1–86.8), with a mean intensity of 380 o.p.g.; in *R. p. ornata* the prevalence was 94.4% (85/90) (95%, C.I. = 89.7–99.2), with a mean intensity of 1,093 o.p.g.

Based on morphological analysis of oocysts, three morphotypes attributable to *E. rupicaprae*, *E. riedmuelleri*, and *E. yakimoffmatschoulskyi* were detected in both chamois species. In addition, *E. suppereri* was recognized in *R. r. rupicapra* from Area B and, noteworthy, two small oocysts recovered from one chamois originated from Area A and consistent with the descriptions of *E. alpina* were identified. Due to the absence of sporulated forms, these two oocysts were excluded from the statistical analysis.

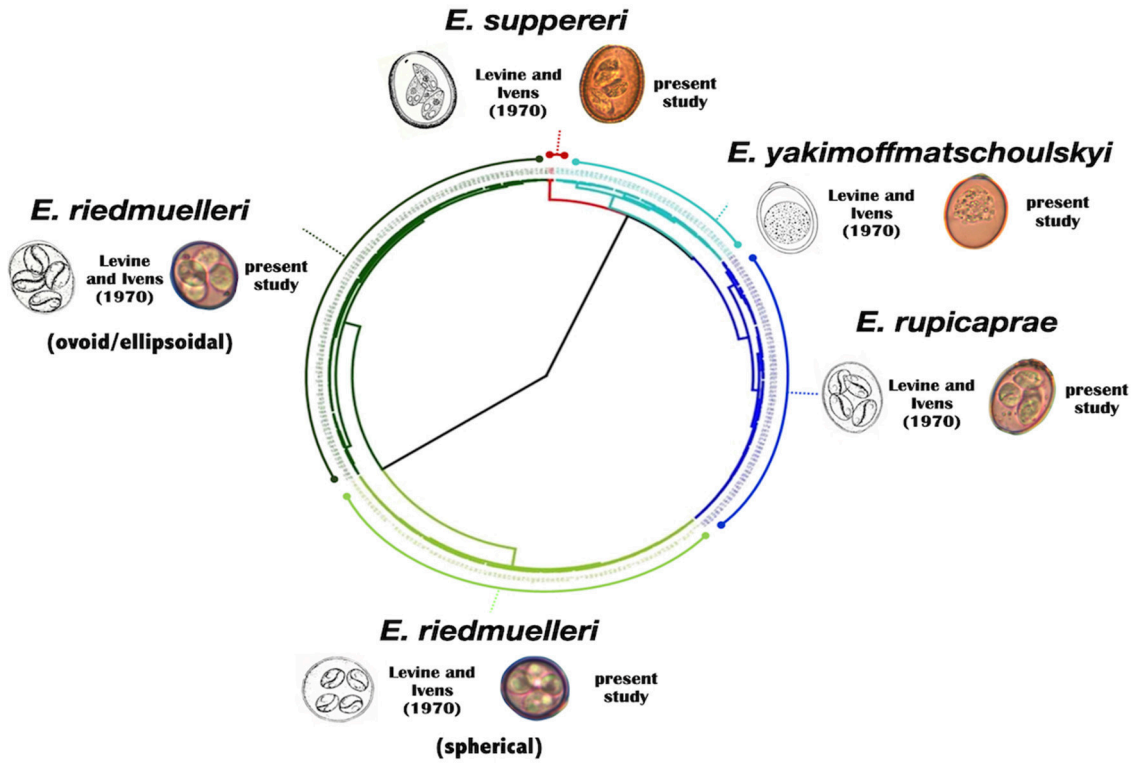

FIGURE 2 | Circular dendrogram issued from the HCPC analysis (output of k = 5) based on length, width and shape index for both *Eimeria* spp. oocysts and sporocysts.

Through the HCPC analysis, five well-defined clusters (k = 5) grouping oocysts from both hosts were generated (see **Table 1** and **Figure 2**). In summary, clustering was as follows:

Cluster 1 (red cluster) gathers the biggest oocysts ($n = 4$) of our dataset, measuring on average 47.54 by 35.38 µm; the mean measures of the sporocysts were 19.48 by 10.88 µm. These isolates were assigned to *E. suppereri*. Cluster 2 (light blue cluster) grouped 37 oocysts measuring on average 29.71 by 21.3 µm. The sporocysts measured 13.74 by 6.92 µm. We attributed these isolates to *E. yakimoffmatschoulskyi*. Cluster 3 (dark blu cluster) includes 75 oocysts measuring 27.02 by 22.33 µm. The sporocysts were 11.75 by 7.71 µm. We assigned these isolates to *E. rupicaprae*. Cluster 4 (light green cluster) consists of 82 spherical oocysts with a mean size of 18.28 by 16 µm. The sporocysts were 7.57 by 5.99 µm. We assigned these isolates to the spherical form of *E. riedmuelleri*. Finally, Cluster 5 (dark green cluster) pools 96 oocysts measuring on average 20.21 by 17.4 µm. The sporocysts were 8.14 by 6.24 µm. These isolates were assigned to the ovoid/ellipsoidal *E. riedmuelleri* form.

A detailed description of morphometrical data is given in **Table 1**.

DISCUSSION

Eimeriid protozoans are common parasites in ruminants worldwide, often associated with enteritis, weight loss and mortality in young animals (17). High prevalence and intensity of infection have been also documented in wild ungulates, where asymptomatic infection largely prevail (18). The high prevalence and intensity of oocysts emission recorded in this study shows that infection by eimeriid protozoa is also widespread amongst members of the *Rupicapra* genus in Italy, confirming previous findings (12, 19). The normal fecal consistency of analyzed samples suggests that infection by *Eimeria* spp. is substantially sub-clinical in both hosts.

To overcome the considerable amount of intraspecific and interspecific variation exhibited in the key morphological features of oocysts, and the drawbacks linked to the presence of multiple infections, as usually occurs in wildlife, in the present study a statistical method was performed for the identification of *Eimeria* spp.. The hierarchical cluster analysis adopted here was able not only to verify the robustness of original taxonomic description of the *Eimeria* species known to parasitize the

members of the genus *Rupicapra,* but, noteworthy, to provide a statistical significance to their morphological variability. Cluster analysis (see **Figure 2**) highlights that distinct forms can be separated based upon their morphology. As reported in Levine and Ivens (4), the measurements obtained from the oocysts grouped in Cluster 1 and 2 overlap unequivocally with the values describing *E. suppereri* and *E. yakimoffmatschoulskyi* according to Restani (11), respectively; Cluster 3, grouping oocysts of *E. rupicaprae* corresponds more strictly to Restani (11) measurements than to those by Galli-Valerio (20, 21) and Yakimoff and Matschoulsky (22). Surprisingly, within the species *E. riedmuelleri*, the splitting of the two well-defined Clusters 4 and 5 was in line with the two morphotypes (the spherical and the ovoid or ellipsoidal oocysts) described by Yakimoff and Matschoulsky (22) and Levine and Ivens (4). Hence, our results based on statistical method strongly support the existence of these distinct morphological groups, possibly referable to two different taxonomic entities infecting chamois, whose identity requires further in-depth investigations.

Remarkably, the Northern and Southern chamois shared most of the *Eimeria* species identified, suggesting that wild Caprines may be a suitable model to explore in depth the amplitude of the host specificity characterizing eimeriid protozoa (23, 24).

The absence of the large-sized, hence easy detectable, *E. suppereri* in the Southern chamois might reflect a possible effect of the life history of the Apennine subspecies of *R. pyrenaica ornata*, characterized by prolonged population bottlenecks (9, 25).

In conclusion, this study provides a deepening into the diversity of *Eimeria* species and highlights the not negligible prevalence of these coccidian protozoan infecting chamois in Italy. The presence of *E. rupicaprae*, *E. yakimoffmatschoulskyi*, *E. suppereri* and of *E. riedmuelleri* in *R. r. rupicapra* and of *E. riedmuelleri, E. rupicaprae* in *R. p. ornata* is confirmed. Moreover, *E. alpina* and *E. yakimoffmatschoulskyi* are additional species of the eimerian fauna of the Northern and Southern chamois. The combination of morphological data with a robust statistical method, as here proposed, represents a useful approach to infer the taxonomy and, consequently, to investigate the epidemiology of these protozoans with the due accuracy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because this research did not involve purposeful killing of animals. Fecal samples in hunting districts were gathered from chamois legally shot by hunters in accordance with the Italian Law (157 of 11/02/1992) which implies that hunters have to carry culled wild ungulates to the control centers where, for each subject, age, sex, the shooting area, and morpho-biometric measures are registered. Thus, no animals were killed specifically for this study.

AUTHOR CONTRIBUTIONS

FB, PL, LR, MMDF, SD'A, and AG participated in study activities and in drafting and revising the manuscript. CDL, IM, NF, TT, NF, and FF participated in the field and laboratory work. MMDF performed the statistical analysis. All Authors have participated in critically revising the manuscript.

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Reactivity of Horse Sera to Antigens Derived From *Sarcocystis falcatula*–Like and *Sarcocystis neurona*

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Sarcocystis neurona and *Sarcocystis falcatula* are protozoan parasites endemic to the Americas. The former is the major cause of equine protozoal myeloencephalitis, and the latter is associated with pulmonary sarcocystosis in birds. The opossum *Didelphis virginiana* is the definitive host of these parasites in North America. Four *Didelphis* species are found in Brazil, and in most reports in this country, *Sarcocystis* species shed by opossums have been classified as *S. falcatula*–like. It is unknown whether reports on *S. neurona*–seropositive horses in Brazil are also derived from exposure of horses to *S. falcatula*–like. The aim of this study was to test the sera reactivity of 409 horses in Brazil using antigens derived from a Brazilian strain of *S. falcatula*–like (Sarco-BA1) and from a North American strain of *S. neurona* (SN138). Samples were examined by immunofluorescent antibody tests (IFATs) at start dilutions of 1:20, and a selected number of samples was tested by Western blot (WB). Sera from 43/409 (10.5%) horses were reactive to *S. falcatula*–like and 70 of 409 (17.1%) were reactive to *S. neurona* antigen; sera from 25 animals (6.1%) were positive for both parasites by IFAT. A poor agreement was observed between the two employed IFATs ($\kappa = 0.364$), indicating that horses were exposed to more than one *Sarcocystis* species. Horse sera evaluated by WB consisted of four sera reactive to *S. falcatula*–like by IFAT, six sera positive to *S. neurona* by IFAT, two sera that tested negative to both parasites by IFAT, and a negative control horse serum from New Zealand. Proteins in the range of 16 and 30 kDa were recognized by part of IFAT-positive sera using both antigen preparations. We concluded that Brazilian horses are exposed to distinct *Sarcocystis* species that generate different serological responses in exposed animals. Antigens in the range of 16 and 30 kDa are probably homologous in the two parasites. Exposure of the tested horses to other *Sarcocystis* species, such as *Sarcocystis lindsayi*, *Sarcocystis speeri*, and *Sarcocystis fayeri*, or *Sarcocystis bertrami* cannot be excluded in the current study.

Keywords: *Sarcocystis* sp., equine, antibody, Western blot, immunoblot

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INTRODUCTION

The coccidian parasite *Sarcocystis neurona* is the major cause of equine protozoal myeloencephalitis (EPM), a debilitating neurological disease that affects horses in the Americas (1, 2). The opossum *Didelphis virginiana* serves as definitive host of *S. neurona* in North America (3, 4), whereas *Didelphis albiventris* was identified as definitive host of *S. neurona* in Brazil (5). While only one species of *Didelphis* is found in North America, four species of this genus are found in Brazil: *D. albiventris*, *Didelphis aurita*, *Didelphis marsupialis*, and *Didelphis imperfecta* (6). The North American opossum (*D. virginiana*) is definitive host of three species of *Sarcocystis*: *Sarcocystis falcatula* (7), *S. neurona* (3), and *Sarcocystis speeri* (8). The South American opossum *D. albiventris* is definitive host of four species of *Sarcocystis*: *S. neurona*, *Sarcocystis lindsayi*, *S. speeri*, and *S. falcatula* (5, 9–11). Under experimental conditions, *S. falcatula* and *S. lindsayi* are infective for birds (11, 12), whereas *S. neurona* and *S. speeri* are infective for immunodeficient mice (13, 14).

Similarly to the protozoan parasite *Toxoplasma gondii*, *S. neurona* also contains several surface antigens (SAGs) which are probably associated with parasite virulence and host cell invasion (15). Three *S. neurona* SAGs (SnSAGs), named as SnSAG2, SnSAG3, and SnSAG4, were identified in merozoites of all *S. neurona* isolates and have been employed in an enzyme-linked immunosorbent assay (ELISA) for EPM in horses (16–18). Coding genes for SAGs from Brazilian isolates of *S. falcatula*–like are very similar to those from North American isolates of *S. neurona* (19). A high allelic variation is found for SAG2, SAG3, and SAG4 from *S. falcatula*–like, contrasting to SAGs from *S. neurona* in North America that possesses low genetic variation (20, 21).

Several serological techniques have been developed to detect *S. neurona* antibodies, including Western blot (WB), immunofluorescent antibody test (IFAT), *S. neurona* agglutination test and ELISA (2). WB using serum and cerebrospinal fluid from horses was the first serological test developed for EPM in horses (22). In the last two decades, IFAT and SnSAG ELISA have been validated for *S. neurona* infections and have been frequently used in veterinary practice and in research investigations (1). WB continues to be a valuable tool on *Sarcocystis* species investigations; however, its application has been essentially in research studies (2).

S. falcatula is a parasite shed by opossums that causes severe respiratory disease in birds (7, 23, 24). Serologic cross-reactivity between *S. falcatula* and *S. neurona* was suspected as some genes coding for immunodominant SAGs for these parasites are very similar (25). In this context, the SnSAG 2-4 ELISA would not be specific for *S. neurona* and could present cross-reactivity for *S. falcatula*–infected animals (25). In an experimental study conducted in the late 1990s, four horses in the United States did not seroconvert after experimental inoculation with *S. falcatula* sporocysts (26); it was assumed that cross-reactivity between *S. neurona* and *S. falcatula* would not be a concern when testing horses by SAG ELISA, as *S. falcatula* would not induce seroconversion in horses (1).

In Brazil, *Sarcocystis* species shed by opossums possess intriguing characteristics. In recent years, more than 50 *Sarcocystis* species isolates were obtained from Brazilian opossums, and almost 100% of them were infective to birds (budgerigars). However, these isolates were genetically distinct from both *S. falcatula* and *S. neurona* and, for this reason, classified as *S. falcatula*–like (20, 21, 27–29). The Brazilian isolates that were submitted for sequencing of SAG genes possessed a high allelic variation in their coding genes for SAG2, SAG3, and SAG4, which seems to represent genetic recombination between *S. neurona*, *S. falcatula*, or other unidentified *Sarcocsytis* species (19–21, 27–29). Based on this peculiar scenario in Brazil, we hypothesized that Brazilian horses may be exposed and seroconvert to other species of *Sarcocystis* shed by opossums, besides *S. neurona*. To address this hypothesis, we tested horse sera using antigen from a North American strain of *S. neurona* and antigen derived from a recently isolate of *S. falcatula*–like, which has been propagated in an avian cell line (27).

MATERIALS AND METHODS

Horse Sera

Serum samples were obtained from 409 adult horses, including males and females, mostly from mixed breeds, and derived from Bahia and Rio Grande do Sul states in Brazil. Samples from Bahia state (n =217) were collected as part of the clinical screening for equine infectious anemia virus and for hematological checking. Samples from Rio Grande do Sul (n =192) were acquired in a commercial slaughterhouse for horse meat exportation. No animals were raised or handled for research purposes. Horse sera were stored for 5 years at −20°C in the Laboratory of Coccidian Protozoa at the School of Veterinary Medicine from Federal University of Bahia.

Merozoites and Antigen Production

Antigens for IFATs and for WB consisted of merozoites of a North American strain of *S. neurona* (SN-138) (30) and merozoites from a South American strain of *S. falcatula*–like (27). *S. neurona* merozoites were propagated in Vero cells supplemented with RPMI-1650 + L-glutamine (Invitrogen/Gibco®, Carlsbad, USA), 1% antibiotic–antimycotic (100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B) (Gibco®, Carlsbad, USA), and 5% of inactivate bovine serum (Invitrogen/Gibco®, Auckland, NZ), at 37°C in a humidified incubator containing 5% CO_2. *S. falcatula*–like merozoites were grown in the same conditions as described above, but instead of Vero cells, the parasites were cultured in a permanent chicken cell line (UMNSAH/DF-1) (31), as recently described (27). Cell monolayers containing merozoites of each parasite species were scrapped from the flasks, passed three times through a 26-gauge needle, filtered in Sephadex G-25 (GE Healthcare®) columns, and washed three times in phosphate-buffered saline (PBS) by centrifugation (1,500 g for 5 min).

TABLE 1 | Seropositivity of Brazilian horses to *Sarcocystis neurona* and *Sarcocystis falcatula*–like tested by immunofluorescent antibody tests.

	S. neurona	***S. falcatula*–like**	***S. neurona* + *S. falcatula–like***
Frequency of seropositive	17.1% (70/409)	10.5% (43/409)	6.1% (25/409)
Frequency of seronegative	82.9% (339/409)	89.5% (366/409)	78.7% (322/409)

Immunofluorescent Antibody Tests

Volumes of 10 μL, containing of 3×10^3 purified merozoites of *S. neurona* or *S. falcatula*–like were added to each 5-mm well of IFAT slides, which were dried at 37°C. Antigen slides were immersed in cold acetone (−20°C) for 10 min for fixation and stored at −20°C until analysis. Antigen-coated slides were stored at a maximum time of 60 days until examination by IFAT.

Serum samples were tested at a starting dilution of 1:20 in PBS. Slides were incubated at 37°C for 30 min in a humid chamber and then washed for 10 min in a FA (fluorescent antibody) buffer (26.9 mM Na_2CO_3, 100 mM $NaHCO_3$, 70.6 mM NaCl, pH 9.0) and 10 min in PBS and dried at 37°C. A fluorescein isothiocyanate–conjugated anti–horse immunoglobulin G (IgG) (Sigma–Aldrich®, St. Louis, USA) was used as secondary antibody at 1:32 dilution and incubated for 30 min in a dark and humid chamber. Slides were washed in FA and PBS as described above, dried at 37°C, and mounted with buffered glycerin. Reactions were observed under a fluorescent microscope (CiL, Nikon®). Positive controls consisted of sera from naturally exposed horses that reacted at 1:80 solely for each parasite (*S. neurona* or *S. falcatula*–like). Negative controls consisted of previously examined horse sera that tested negative for both parasites at dilutions <1:20. Positive reactions were characterized by full peripheral fluorescence of merozoites. Antibody titers were determined by double dilutions for all reactive sera.

WB/Immunoblot

The term WB (Western blot) is used here and throughout the manuscript to indicate both WB and immunoblot. Cultured merozoites of *S. neurona* (4×10^7 per membrane) or *S. falcatula*–like (2×10^7 per membrane) purified in Sephadex G-25 columns (in *Merozoites and Antigen Production*) were pelleted by centrifugation and mixed with a reducing sample buffer (1% 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 7% glycerol, 48 mM Tris–HCl, pH 6.8), heated at 97°C for 10 min, and centrifuged at 13,000 g for 10 min at 4°C.

WB was performed similarly as previously reported (32). The solubilized proteins from merozoites were run on a 12.5% polyacrylamide gel electrophoresis with SDS. A prestained molecular weight marker with proteins from 10 to 180 kDa (PageRuler Prestained Protein Ladde, Thermo Scientific™) was used on each gel. Proteins were transferred from the gels to polyvinylidene difluoride (PVDF) membranes, blocked with PBS–Tween–gelatin (0.05% Tween 20 and 2% of gelatin) for 30 min, and stored at −20°C until analysis.

For immunoblot, each PVDF membrane coated with *S. neurona* or *S. falcatula*–like antigen was cut in 26 strips. Sera from 13 horses were selected for the analysis, including a negative control (horse serum from New Zealand), two horse sera that tested negative for both parasites by IFAT, six samples that tested positive for *S. neurona* by IFAT, and four sera that tested positive for *S. falcatula*–like by IFAT. Reactions were conducted in two different ways: 1) using antigen strips that were blocked with bovine serum containing antibodies to *Sarcocystis cruzi*, as reported by Rossano et al. (33); 2) using antigen strips not treated with anti–*S. cruzi* serum. Serum containing antibodies to *S. cruzi* was obtained by testing bovine sera with bradyzoites' antigen by IFAT (34).

Membrane strips from both parasites were blocked for 90 min with anti–*S. cruzi* bovine serum diluted at 1:65 (33) in PBS–Tween–gelatin. After five washings with 0.05% Tween-20 in PBS (PBS-T), membrane strips were incubated at room temperature with horse sera at 1:10 in PBS-T gelatin for 60 min. Then, they were incubated with anti–horse IgG peroxidase conjugate for 60 min and washed three times with PBS-T and three times with PBS. The reactions were revealed using diaminobenzidine peroxidase tablets and stopped by adding ultrapure water. The same 13 horse sera were tested by WB to both parasite antigens without initial incubation with anti–*S. cruzi* bovine serum.

Statistical Analysis

To compare the percent agreement in IFATs for *S. neurona* and *S. falcatula*–like, Cohen κ statistics was used. Characterization of labeled bands in WB was determined by descriptive statistics, by means of the frequency of the observed bands.

RESULTS

Immunofluorescent Antibody Tests

Antibodies to *S. neurona* (SN138 strain) and to *S. falcatula*–like (Sarco-BA1 strain) antigens were detected by IFAT in 17.1% (70/409) and in 10.5% (43/409) of the horses, respectively. A 1:20 dilution was used for the study. Simultaneous seropositivities for both antigens were observed in 6.1% (25/409) of the animals. A total of 322 (78.7%) of 409 horses were seronegative to both parasites. The agreement level for the two IFATs, expressed by the κ coefficient, was 0.364, indicating a fair agreement between the two tests. IFAT results are shown in **Table 1**. The maximum antibody titers observed for the two tested antigens was 1:160; 11 animals were positive at 1:160 for *S. neurona*, and 2 animals for *S. falcatula*–like presented titers of 1:160 by IFAT (**Supplementary File**).

Western Blot

In WB, reduced antigens from both parasites were tested using sera from 13 horses, including a negative control, two horses that tested negative to both parasites by IFAT, six horses that tested

TABLE 2 | Horse sera selected for Western blot and their antibody titers for *Sarcocystis neurona* and *Sarcocystis falcatula* determined by IFAT.

Animal ID	Antibody titer to *S. neurona*	Antibody titer to *S. falcatula*–like
1	Neg	Neg
2	Neg	Neg
3	Neg	Neg
4	1:160	Neg
5	1:80	Neg
6	1:80	Neg
7	1:80	Neg
8	1:80	Neg
9	1:80	Neg
10	Neg	1:40
11	Neg	1:20
12	Neg	1:40
13	Neg	1:20

positive to *S. neurona*, and four horses that tested positive to *S. falcatula*–like by IFAT (**Table 2**).

Most samples showed varying levels of reactivity to molecules in the regions of 16 and 30 kDa, regardless of *S. neurona* (**Figure 1A**) or *S. falcatula*–like (**Figure 1B**) were used as antigens in WB. The reactive bands in the antigen strips treated with anti–*S. cruzi* serum did not show significant differences from those not treated with anti–*S. cruzi* serum. Labeling of several antigens, besides those in the range of 16 and 30 kDa, were visualized between 10 and 70 kDa, with apparently no diagnostic value.

DISCUSSION

To test the hypothesis that Brazilian horses may be exposed and seroconvert to more than one *Sarcocystis* species shed by opossums, we examined horse sera from Bahia and Rio Grande do Sul states using two *Sarcocystis* species as antigens. Merozoites from a North American strain of *S. neurona* (SN-138) and from a South American strain of *S. falcatula*–like (Sarco-BA1) were employed as antigens in IFAT and WB. All samples were examined by IFAT, and 13 selected sera were tested by WB using antigens from both parasites. The sera tested by WB were processed with and without a blocking step with bovine serum anti–*S. cruzi*.

Results obtained by IFAT indicated that the examined horses reacted to more than one species of *Sarcocystis*; the κ coefficient was 0.364, supporting that the frequency of seropositivity in the IFAT for *S. neurona* had a fair agreement with the IFAT for *S. falcatula*–like. A total of 45 horses reacted solely to *S. neurona* antigen by IFAT, and 18 horses showed positive reactions solely to *S. falcatula*–like. A starting serum dilution of 1:20 was selected for the current study because in a previous work, a gold standard panel of horses infected with *S. neurona* had seropositivities between 1:20 and 1:80 by IFAT (35); these authors recommended a 1:80 cutoff in IFAT for horses suspected to have EPM. In the current work, equine sera were derived from horses with no neurological disease, and for this reason, we decided to use a less conservative cutoff.

The labeling patterns in WB using antigens of *S. neurona* and *S. falcatula*–like were very similar, indicating serological cross-reactivity for several shared antigens. The WB reactions using a blocking step with bovine anti–*S. cruzi* serum to minimize nonspecific reactions did not lead to any significant change in the results. Slight differences were observed in the intensity of the labeled antigens; however, it may be related to the time that enzymatic reactions were stopped during immunoblot. In literature, antigens regarded as immunodominant for *S. neurona* infection in horses possess approximate molecular weights of 16 and 30 kDa (33). Some authors also include proteins in the range of 7 to 10 kDa, besides the 16- and 30-kDa antigens as specific for *S. neurona* exposure (36). Positive reactions to one of the two proteins (16 or 30 kDa) are considered suspect for *S. neurona* infection (35). In subsequent studies with larger sample sets, the main concern of WB for *S. neurona* in horses corresponded to its low specificity (35, 37, 38).

Several reports on *S. neurona* infections or exposure in Brazilian animals have been conducted using North American strains of the parasite (39–43). It is worth to note that *S. neurona* antigen derived from Brazilian isolates of the parasite is not available for serological tests. The opossum species identified as definitive host for *S. neurona* in the United States (*D. virginiana*) does not exist in Brazil. The only description of *S. neurona* in a Brazilian opossum (*D. albiventris*) was reported in 2001 (5); parasite identification was mainly based on its infectivity to immunodeficient mice and by polymerase chain reaction–restriction fragment length polymorphism according to primers designed by Tanhauser et al. (44). At the time *S. neurona* was described in *D. albiventris* (5), the employed molecular tools were believed to precisely identify the *Sarcocystis* species infecting the opossum. In recent years, it became clear that additional molecular techniques are needed to differentiate *S. neurona* from closely related *Sarcocystis* species shed by opossums, including *S. falcatula*–like organisms (27).

In a recent publication, a Brazilian cat was reported to have *S. neurona* infection (45); based on the internal transcribed spacer 1 (ITS1) of the rDNA, this *Sarcocystis* species differed from organisms classified as *S. neurona* or *S. falcatula*. These authors used additional molecular markers, including *SAG* loci, *18S*, and *COX1*; the combined molecular data, mostly based on ITS1 and *SAG* loci, allowed the classification of this cat isolate as *S. neurona*, although it is clearly distinct from the North American isolates of *S. neurona* (45).

Studies on Brazilian *Sarcocystis* species shed by opossums, based on bioassay and different molecular markers, revealed that all isolates differed from *S. neurona*, in both biological and molecular aspects (19–21, 27, 29); the reported isolates were infective to budgerigars and possessed a high level of genetic recombination. In light of the peculiar scenario observed in Brazil, some questions have been raised by our research group: 1) May horses be infected with or present seroconversion to *S. falcatula*–like? 2) Are there other *Sarcocystis* species shed by opossums that are capable of infecting horses in Brazil? Although

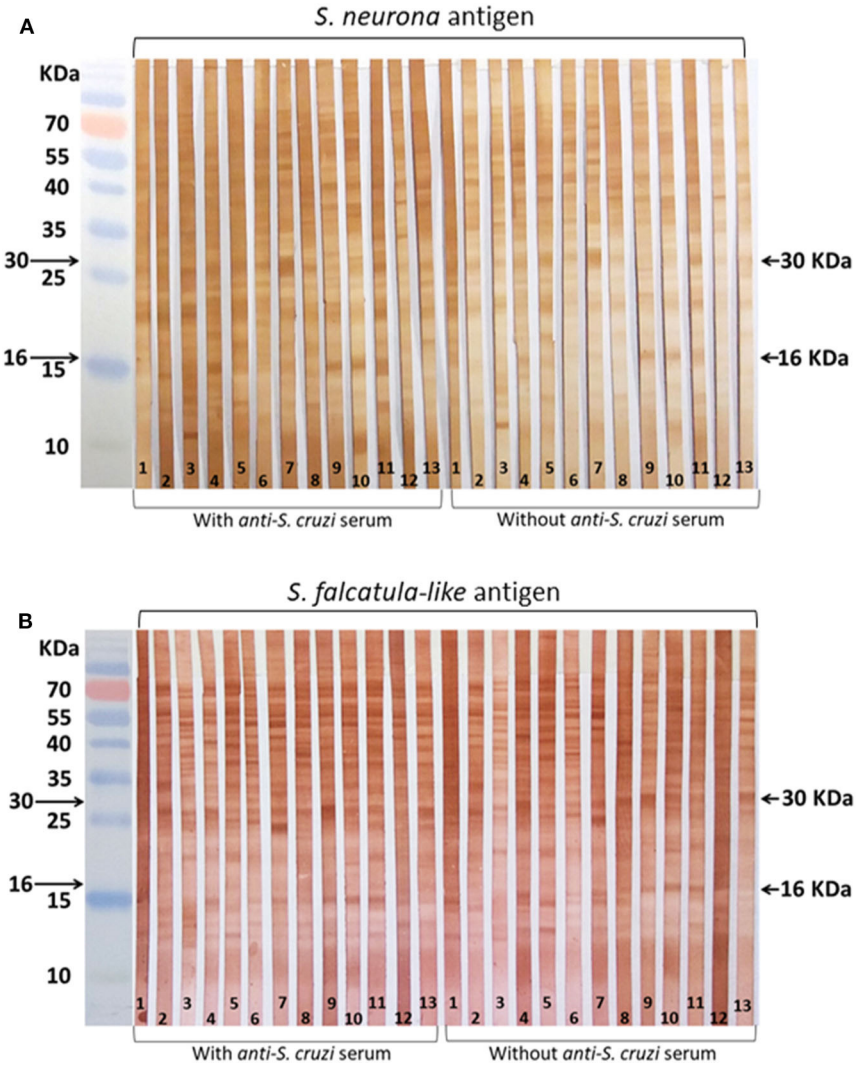

FIGURE 1 | Horse sera tested by Western blot (WB). Serum samples were selected for WB by immunofluorescence antibody tests (IFATs). **(A)** Membrane strips coated with *Sarcocystis neurona* antigen; **(B)** membrane strips coated with *Sarcocystis falcatula*–like antigen. Reactions were conducted with and without blocking treatment with bovine anti–*Sarcocystis cruzi* serum. Strip 1: negative control (horse serum from New Zealand); 2–3: sera that tested negative for both parasites by IFAT; 4–9: sera that tested positive for *S. neurona* by IFAT; 10–13: sera that tested positive for *S. falcatula*–like by IFAT.

EPM associated to *S. neurona* was reported in Brazilian horses, identification of the parasite was mainly based on clinical, morphological, and immunological tests, including serology and immunohistochemistry (46, 47). So far, there is no isolation or molecular identification of *S. neurona* from any Brazilian horse. Serological studies in the country have been conducted with North American *S. neurona* antigens, in both IFAT and ELISA. In a serological survey performed with Brazilian horses from several states, the overall frequency of *S. neurona* antibodies was 69.6% (669/961) using an ELISA with SAG4 as antigen (40). Interestingly, in two recent seroepidemiological studies in Brazil using IFAT, frequencies of antibodies to the parasite were 26% ($n = 506$) in Minas Gerais (48), and 2.8% ($n = 427$) in the state of Alagoas (49); in these two studies, North American isolates of *S. neurona* were used with a 1:80 cutoff. In the present study, we detected 17.1% ($n = 409$) of seropositive animals by IFAT using *S. neurona* as antigen and 1:20 as cutoff. The differences in seropositivities between SAG4 ELISA (69.6%) and IFAT (2.8–26%) for Brazilian horses raise suspicions that results were overestimated by using SAG4 ELISA or underestimated by using IFAT. Inclusion of SnSAG ELISA (17, 18) in the current study, as well as a sample set of sera from horses with confirmed *S. neurona* infections, would highly aid on evaluation of cross reactions between anti–*S. neurona* antibodies and *S. falcatula*–like antigens. Performing immunoblotting with *Neospora* spp. antigens would also contribute to test potential cross-reactivity between this genus and *Sarcocystis* spp.

In the current work, we detected horses that reacted solely to *S. falcatula*–like antigen by IFAT and horses with positive

reactions solely to *S. neurona* antigen by IFAT. For this reason, we suspected that more than one species of *Sarcocystis* species shed by Brazilian opossums induce seroconversion in horses. In a recent study, we performed experimental infection in Mongolian gerbils using *S. neurona* and *S. falcatula*–like (50). Serological cross-reactivity between the two parasites was clearly demonstrated by WB, whereas IFAT was able to distinguish infections caused by each of these parasites (50).

In conclusion, we demonstrated that using IFAT as serological test, Brazilian horses reacted differently to *S. neurona* and *S. falcatula*–like antigens. A 1:20 cutoff was employed in each IFAT; however, the optimal cutoff was not determined in this study, as no gold standard sera are available for Brazilian horses. Seroconversion of the tested animals to other *Sarcocystis* species that infect horses, including *Sarcocystis bertrami* or *Sarcocystis fayeri* (51), as well as to other *Sarcocystis* species shed by opossums, such as *S. lindsayi* or *S. speeri*, cannot be excluded in the current work. It is crucial to conduct further studies on molecular identification of *Sarcocystis* species in Brazilian horses with EPM, as well as to determine whether Brazilian strains of *S. falcatula*–like induce seroconversion in horses by oral ingestion of sporocysts of the parasite. To our knowledge, this is the first study to test horse sera with *S. falcatula*–like antigens and to provide evidence of serologic cross-reactivity in horses involving *S. neurona* and *S. falcatula*–like. Further studies are needed to determine an appropriate serological test to aid on diagnosis of EPM in Brazilian horses.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because no animals were raised or handled for research purposes, therefore, no license was required for the experiments. Samples from Bahia state ($n = 267$) derived from regular clinical screening for equine infectious anemia virus and for hematological checking. Samples from Rio Grande do Sul state ($n = 142$) were acquired in a commercial slaughterhouse for horse meat exportation.

AUTHOR CONTRIBUTIONS

WB-S: analysis and drafting of the manuscript. RJ: Western blot analysis and revision of the manuscript. RF: assistance in cell culture and IFAT. LG: designed the experiment and revised the manuscript drafts. All authors approved of the final version of the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.573016/full#supplementary-material

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REVIEW
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Possibilities for IPM Strategies in European Laying Hen Farms for Improved Control of the Poultry Red Mite (*Dermanyssus gallinae*): Details and State of Affairs

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The Poultry Red Mite (PRM), *Dermanyssus gallinae,* is a major threat to the poultry industry worldwide, causing serious problems to animal health and welfare, and huge economic losses. Controlling PRM infestations is very challenging. Conventionally, *D. gallinae* is treated with synthetic acaricides, but the particular lifestyle of the mite (most of the time spent off the host) makes the efficacy of acaracide sprays often unsatisfactory, as sprays reach only a small part of the population. Moreover, many acaricides have been unlicensed due to human consumer and safety regulations and mites have become resistant to them. A promising course of action is Integrated Pest Management (IPM), which is sustainable for animals, humans and the environment. It combines eight different steps, in which prevention of introduction and monitoring of the pest are key. Further, it focusses on non-chemical treatments, with chemicals only being used as a last resort. Whereas IPM is already widely applied in horticulture, its application is still in its infancy to control *D. gallinae* in layer houses. This review presents the currently-available possibilities for control of *D. gallinae* in layer houses for each of the eight IPM steps, including monitoring techniques, established and emerging non-chemical treatments, and the strategic use of chemicals. As such, it provides a needed baseline for future development of specific IPM strategies, which will allow efficient and sustainable control of *D. gallinae* in poultry farms.

Keywords: *Dermanyssus gallinae*, integrated pest management, poultry red mite, sustainable control, prevention, non-chemical, monitoring, layer houses

INTRODUCTION

The Poultry Red Mite (PRM), *Dermanyssus gallinae* (De Geer, 1778) (**Figure 1**), is the most common ectoparasite belonging to the order of Mesostigmata and class of Arachnida (1) in poultry farming. A taxonomic key and high-resolution micrographs are available for a correct identification of the species (2, 3). It is a blood-sucking ectoparasite of laying hens, living off the host and mainly

hiding in cracks and crevices near the hen's nightly resting place, therefore out of reach of the hens (4, 5). However, as it is a strictly hematophagous mite, it needs to find a hen from time to time to obtain a fast blood meal, which it does during the dark hours (6).

Dermanyssus gallinae is the most damaging ectoparasite of laying hens worldwide, particularly in Europe where the infestation rate of premises is well over 80% and in some countries even reaching 94% (7). Due to relatively high

FIGURE 1 | Photograph of an *in situ* mite (*Dermanyssus galllinae*) aggregate, composed of different stages of *D. gallinae* more or less freshly fed. The mite droppings (black and white marks surrounding the aggregate) form fairly persistent marks on structural elements in the farms, lasting long after an infestation. Photo credit: Rumsais Blatrix (CEFE/CNRS).

temperature and humidity, environmental conditions in a layer house are in general very favorable for *D. gallinae*. In such favorable conditions, the reproduction cycle of *D. gallinae*, can be completed in only 7 days, which often leads to a rapid exponential accumulation to very high numbers in a short period of time (8, 9). Infestations of *D. gallinae* pose serious threats to the welfare of the hens. They may cause restlessness, lack of sleep, stress, severe feather pecking, aggression, anemia and sometimes even death (1, 5, 10–13). Further, *D. gallinae* also poses a threat to human health, as it can act a reservoir and possibly as a vector of several zoonotic diseases like *Salmonella enteritidis, Pasteurella multocida,* and *Borrelia burgdorferi* (8, 11). The ectoparasite also increasingly causes human dermatological lesions in poultry handlers and urban citizens, which is currently still largely underdiagnosed (7, 12, 14). Infestations of *D. gallinae* also lead to huge economic losses. First of all, there is an increased mortality. Further, infested flocks have a higher feed and water conversion due to poor feathering and loss of blood. Despite the increased feed intake, hens will be lighter and less energy will go to the eggs, leading to lower production and lower egg weight. In addition, the percentage of second quality eggs increases due to a.o. blood spots (15, 16). Based on literature and field experience, Van Emous (2005) estimated the economic damage of *D. gallinae* infestations. He adapted these figures in 2017 (**Table 1**), as the situation changed mainly due to alternative housing systems, extended production cycles, and ban on beak-trimming in several countries (leading to more severe pecking impact). Including treatment costs, he estimated an economic damage of 31 million euros per year associated with *D. gallinae* in Europe.

TABLE 1 | Estimation of economic impact at different levels of infestations of *D. gallinae*: medium (mites not visible); severe (mites visible); and very severe (many clusters visible on the system), according to (17), and updated for a severe infestation by Van Emous (15).

	None	Medium	Severe	Very severe
Feed intake (g/day/hen)	108	+0	+2	+2
Egg weight (g)	62	−0.2	−1	−1
Hen weight (g)	1,800	−25	−100	−100
Second q. egg (%)	6	+2	+6	+14
Mortality (%)	7	+0	+2	+5
Number of eggs (per hen housed)	345	−0	−3	−10

CURRENT CONTROL STRATEGIES

Conventional treatment of *D. gallinae* is dominated by the use of synthetic acaricides. However, there is currently a very limited amount of chemical acaricides available for use, as many have been withdrawn from the European market due to human consumer and user safety regulations (8). Further, increasing levels of resistance to commonly applied acaricides has been found in *D. gallinae*, causing lower efficacy of these products (18). The solitary use of chemicals is thus an end-of pipeline solution which is not regarded as sustainable (19). Much research has been done, and is still being done on the use of alternative non-chemical treatments, like plant-based products, natural predators and vaccines (see below *Non-chemical Treatment Methods*). However, none of these seem to be efficient enough to serve as a stand-alone treatment against *D. gallinae* (16). The only sustainable solution to control *D. gallinae* infestation is to use multi-tactic Integrated Pest Management programmes, as already suggested by several authors (5, 20–23). However, in practice, use of this approach in the poultry industry is still very limited and merely restricted to a combination of biosecurity measures, chemical acaricides and physical treatments in-between flocks (9, 23).

INTEGRATED PEST MANAGEMENT

Integrated Pest Management (IPM) is a strategy to control pest species, which is sustainable for animals, humans, and the environment. IPM consists of eight steps, in which prevention of introduction, and monitoring of the pest are key for sustainable control (24). For successful IPM, all ecological and biological knowledge, including biotic and abiotic factors, of the pest species should be integrated. Monitoring is crucial to identify the best moment for applying treatments. Principally, environmentally-safe, non-chemical methods and measures are used for prevention and control of the pest species. Only when non-chemical measures have failed and an action threshold is exceeded is a chemical treatment deployed as a last resort. Preferably, a selective acaricide should be used in order to avoid killing non-target species, and the use of chemicals should be in an as limited way as possible (e.g., hot-spot treatments). Actions to avoid resistance against products should be implemented, and finally, thorough evaluation of the IPM strategy is needed to optimize it.

In horticulture, IPM is currently well-advanced and widely applied. Already since 1959, the International Organization for Biological and Integrated Control (IOBC) has formed international, multidisciplinary teams, to examine methods for biological controls, each team focusing on a particular pest. Through the work of the IOBC, specific IPM strategies for all major crops have been developed. Currently, all EU countries need to have an IPM action plan for these crops (25).

At present, IPM is primarily used to control plant pests, and the practical implementation of IPM in animal husbandry is in its infancy compared to horticulture. Monitoring is only applied in a minority of farms and, in the use of non-chemical alternatives, livestock farming is lagging far behind. Indeed, the definition of biocontrol on governmental or academic webpages often only address plant protection [e.g., (26)].

The potential of IPM in livestock production has already been discussed in 1981 by Axtell. Control of flies, ticks and worms via IPM in dairy farming and pig production is suggested and discussed in several studies (27, 28). Axtell (29) also suggested IPM to control poultry pests, and specifically for the control of *D. gallinae,* IPM has been suggested in several works (5, 8, 19). However, practical implementation is limited to only combining certain treatments (30).

A lot of synergies exist between the arthropod pest control in horticulture and the control of *D. gallinae* in layer farms, so we believe that the principles of IPM can also be applied in the poultry industry. In horticulture, multiple pests are generally tackled simultaneously with IPM strategies. In European layer houses, however, *D. gallinae* seems to be the only problematic ectoparasite, and other targeted pests are mainly flies and lesser mealworm (5). In the US, another problematic ectoparasite in layer houses is the Northern Fowl Mite *Ornithonyssus sylviarum* (Canestrini and Fanzago, 1877), but this pest does not seem to occur in European layer houses (31). Therefore, in contrast to the multiple species targeted in horticulture, the current review focusses on the control of *D. gallinae* solely. In the following sections, we provide an overview and discussion of the currently-available possibilities for each of the eight IPM steps for controlling *D. gallinae* in layer houses, and the still existing deficiencies that limit the delineation and implementation of IPM plans. As such, it provides a needed baseline for researchers to develop valuable research programs for advancing implementation of IPM strategies for control of *D. gallinae*.

TABLE 2 | Cleaning actions to be executed during the empty period for optimal control of *D. gallinae* infestations in a layer house according to Mul et al. (41).

	Actions (preferable executed in this order)
1	Remove manure
2	Remove all clustered manure residues (scraping)
3	Dry clean house (e.g., broom and remove all detritus and dirt)
4	Clean with compressor (also in pvc tubes and cable ties)
5	Clean air mixing box
6	Dry clean hen house second time
7	Clean ventilation duct (preferably with steam cleaner)
8	Clean aeration tubes (possibly by sewer cleaning company)
9	Clean manure belts
10	Clean central manure belts
11	Clean egg belts with high water pressure
12	Remove all dirt from the house
13	Clean whole house with steam cleaner
14	Let everything dry
15	Clean manure container/pit
16	Disinfect after drying

PREVENTION AND POPULATION SUPPRESSION (STEP 1)

Infestations of *D. gallinae* do not only occur in layer farms but also in the breeder flocks and rearing farms, and *D. gallinae* can survive and spread through pullet, egg and manure transport (19, 32).

In layer houses, the very first step in the IPM approach is thus preventing new populations of *D. gallinae* from entering and spreading in the layer houses. Good biosecurity measures are considered highly beneficial for pest control, including *D. gallinae* (33–35).

In 2009, the Hazard Analysis and Critical Control Points (HACCP) method was used as a method for assessing risks in introduction and spread of *D. gallinae* in poultry farms (36). A total of 41 possible hazards for *D. gallinae* infection and spread have been identified by experts, 31 of them being identified as Critical Control Points (CPPs), i.e., points, steps or procedures with a high risk of mite infection and/or spread [(36), Table 2 therein]. These CCPs should be monitored carefully, and possible corrective actions should be undertaken when necessary. The influence of wild birds as a reservoir, as mentioned in this checklist, is, however, currently obsolete, and transmission of *D. gallinae* most probably occurs through the exchange of contaminated material or birds between farms or facilities (37, 38).

Although little information is to be found in the scientific literature, some practical guidelines [brochures and checklists, e.g., (39, 40), MSD Animal Health] for good hygiene and other biosecurity measures are available to poultry farmers for preventing infestation of *D. gallinae* and suppressing its population growth. In a recent project (41) on the control of *D. gallinae,* much attention is payed to these preventive and suppressive measures. The most important measures [based on (41)], are further discussed in the following paragraphs.

Preventing *D. Gallinae* From Entering and Spreading in Laying Hen Facilities

It has been demonstrated that commercial exchanges of contaminated birds and material are strongly involved in the spread of *D. gallinae* in layer farms (37, 38). There are several ways through which *D. gallinae* can enter and disperse throughout the facilities (also between layer houses if there are multiple at the farm). Good biosecurity measures can, however, considerably reduce these chances. Further, existing populations should be prevented to grow to substantial numbers. Preventive measures need to be taken into account during the production as well as between laying rounds, but also when building/installing new facilities.

Between Laying Rounds

When layer houses are empty, hiding places for *D. gallinae,* which cannot be reached easily in the presence of hens, can be cleaned thoroughly. Cleaning with hot water and soap is strongly advised over only dry-cleaning the facilities. **Table 2** illustrates all the steps that should ideally be undertaken, preferably in that order.

Other preventive treatments between flocks can be heat treatment alone or used in combination with inert substances (see further). Heat treatment cannot be used during production as it affects the hens, and thus can only be applied in-between flocks. A temperature of over 45°C is lethal for *D. gallinae* (19, 42). With the Thermokill method, layer houses are gradually heated up to over 45°C for at least 2 days (43). By heating up the layer house gradually, mites are also lured out of their hiding places. This way they are also more reachable with contact treatments (44). Preliminary findings from a field trial showed a good efficacy of applying heat treatment followed

by a silica treatment, with strongly reduced infestation in the next flock (41). A downside of heat treatment seems to be that not all housing systems can resist the high temperatures, resulting in damaged structures. In addition, heat treatment is rather expensive, certainly for larger infrastructures with outdoor facilities for the hens, where heating evenly is difficult (19).

Acaricidal chemicals can also be sprayed during the empty period, but in the framework of IPM, chemical/synthetic treatments should not be used preventively; they should only be used when non-chemical preventive and curative treatments do not act sufficiently.

A project where in 20 commercial layer farms *D. gallinae* was monitored for 10 months (45) showed that the point at which *D. gallinae* is noticed for the first time during a flock is highly influenced by the cleaning and treatments during the empty period. In farms where a combination of dry cleaning, wet cleaning and some form of treatment (silica, plant-based oils, heat treatment or chemical treatments) was applied, a later re-infestation of *D. gallinae* was found compared to the other farms with less stringent regimes.

During Production

Mites are primarily dispersed into the facilities through the actions of people. In a poultry farm, several different people often need to enter the facilities (care-takers, farm managers, veterinarians, etc.). A first measure is to keep the number of external visitors limited and to apply strict hygiene regulations (e.g., wearing company clothing and hair nets, using hygiene barriers, wear separate boots in separate houses) for staff members and visitors. It is also important that delivered pullets and the crates in which they are delivered are mite-free. It is strongly advised that pullet breeders also monitor infestations in their facilities and share their monitoring data with the egg producer. It is also important that egg-trays and egg-containers, which are regularly brought in and out the facilities, are free of mites. Using disposable cardboard egg-trays is preferred. *Dermanyssus gallinae* can also disperse via vertebrates other than hens. A good pest-control strategy (against e.g., mice and rats) is therefore essential. Pets should not be allowed in and in the vicinity of the layer houses. *Dermanyssus gallinae* can also survive on cadavers (46), so these should be removed as fast as possible, at least daily, and preferably transported in sealed bags or clean buckets. A cadaver storage room in the vicinity of the layer houses or against the outside wall of the laying hen facility should be avoided and it should be regularly cleaned and disinfected after removal of the cadavers from the premises.

The infrastructure and nearby environment of the poultry farm are factors which may contribute to reinfection of *D. gallinae* and increased population growth. As storage of manure is also a source of (re)infection, it is best to have it far from the layer houses. *Dermanyssus gallinae* thrives in environments with a lot of crevices so it is therefore important to have all cracks and crevices tightly sealed and open tubes are to be avoided. Smooth materials and environments do not favor mite proliferation. In multiple housing systems, separate tools in different houses should be used to prevent dispersion. All infrastructure that is not easy to clean should be avoided.

Suppressive Measures

During the production cycle, several actions or treatments can be applied to prevent the present mite population from growing to significant numbers. Good hygiene measures have been shown to have such suppressive effects on the mite populations (33, 47). One of the most important actions in housing systems with manure belts (enriched cages and multi-tier aviary systems) is the regular removal of manure. A test at 20 commercial layer houses (41) showed that removing manure more frequently (six times a week instead of 1–2 times a week) resulted in a significantly reduced relative growth rate of the *D. gallinae* population per house, with a higher reduction seen with higher number of mites (−79% reduced growth) of the initial population when compared to the reduction of a lower number of mites of the initial population (−53%). Cleaning the manure belts could also help, certainly if the same manure belt goes through multiple houses. Some other management actions applicable in all housing systems and enabling control of *D. gallinae* are removing dust, manure, and egg debris accumulations regularly.

Control means that can be used proactively to suppress the population growth during a production cycle are: an electrified perch (Q-perch®, to be installed before the arrival of the flock), predatory mites, and repeated treatments with inert substances (e.g., silica). Plant-based feed additives can be administered to sustain the health of the hens. Other treatments are still in development and might also be used as preventive measures in the future: vaccines and entomopathogenic fungi. All possible treatments, established or in development, are discussed more in detail in further sections.

MONITORING POPULATION (STEP 2)

Monitoring Tools

An essential step in IPM strategies against *D. gallinae* is adequate monitoring of the population. As *D. gallinae* is an ectoparasite which does not live on its host, the size of the population is not easily estimated. Furthermore, *D. gallinae* hides in cracks and crevices, thus the major part of the population is mostly hidden (23). Monitoring of *D. gallinae* is, to date, not often implemented in commercial layer farms. However, without proper monitoring tools, mite infestations are only noted when aggregations are visible, when there are blood spots on the eggs and/or when workers perceive irritation or bites. When these signs appear, the infestation is already rather heavy, and it is usually too late for a successful control (19, 48). Monitoring techniques should be able to determine population dynamics and spatial distribution; detect low numbers of *D. gallinae*; determine the effect of treatments; provide knowledge about the population on-farm; detect when a critical threshold for treatment has been reached; and should be species-specific (49, 50). Various different tools have been developed to monitor populations of *D. gallinae* in layer houses (**Table 3**). The most popular, commonly used, or promising methods are discussed in detail below.

In the Mite Monitoring Score (MMS) (57), no mite traps are used. Here, a visual perception of the mite infestation is performed on different points at different levels in the layer

TABLE 3 | List of main techniques for monitoring poultry red mites [adapted from (23) and (50)].

Monitoring method	References
ADAS© Mite Monitor	(ADAS Ltd, Oxon, UK)
Perch trap	(51)
PVC pipe with 13 holes and towel sheet inside	(52)
Tube containing fabric or cloth	(33)
Corrugated cardboard trap	(53)
Tube trap with a wooden stick (Rick Stick) or corrugated cardboard (Avivet)	(54)
Detecting PRM in dust feathers and impurities	(55)
Examining dried droppings for PRM presence	(56)
Folded paper	(56)
Visual Mite Monitoring Score (MMS)	(57)
Velcro band mite trap (MTT)	(58)
Lohmann Trap	(59)
Modified trap after Safrit and Arends	(60)
Semi-Attractive Trap (SAT)	(48)
Simplified Passive Trap (SPT)	(61)
Scout box app	Cropwatch BV
Automated Mite Counter	(23)
Q perch counter (spinn-off from the Q-perch)	(62)
Paper tube trap	(63)
Plastic containers with heating pads	(64)
AviVet trap	(50)

houses. At each point, an area of 1 m^2 is observed and a score of 0–4 is given to estimate the infestation rate.

In other techniques, mites are also assessed visually but tools are used to trap the mites and score the mites in/on these traps. With the Rickstick method (54), a 12 cm long wooden stick is placed into a 10 cm long PVC tube which is attached under the perch. For scoring the infestation, the wooden stick is pulled out of the tube and the number of mites is visually assessed with reference images, also with a score of 0–4. With the semi-passive trap (SPT) (48, 61), mites are scored visually after trapping them with a piece of adhesive tape. A 4-state rating (Score 0–3) and two 2-state ratings (mites absent/present or <10 mites/>10 mites) have been proposed.

In some other monitoring techniques mite numbers are assessed quantitatively, mostly also with the use of traps. Corrugated cardboard traps, as developed by Nordenfors and Chirico (65), are one of the most popular. Here, corrugated cardboard is put inside plastic/PVC tubes that are attached in the housing system (50, 54). Before counting, the traps are usually collected in sealed plastic bags and frozen at −18 to −20°C for at least 2 days to kill the mites. To count the mites, they are all collected from the plastic bag, trap, and dismantled cardboard in a Petri dish (50, 65). After collection, the mites can be counted either manually or with computer-based estimations [e.g., using the program ImageJ, (66)] after taking a photograph (50). Cardboard traps can also be impregnated with acaricides or non-chemical control means like plant-based products or entomopathogenic fungi (see further) to use them as a control mechanism instead of solely as a monitoring tool [(67–69)].

Schulz (60) developed a monitoring trap consisting of a blood test tube filled with corrugated cardboard and compared her trap with the visual MMS method at 16 houses. With the method of Schulz (60), the presence of *D. gallinae* in the layer house was detected earlier than with the MMS method. Moreover, only one of the three mite-free houses according to the MMS method was also mite-free according to the method of Schulz (60). No correlation was found between the MMS score and the number mites per trap.

Mites can communicate with each other (70), signaling others that they have found a suitable hiding space, i.e., the cardboard trap. Therefore, the number of mites in those traps does not correspond accurately with the number of mites effectively present at the place of the trap. This issue is resolved in the use of Semi Attractive Traps (SAT) (48), where mites are attracted to traps with water. Here, small plastic jars with perforated lids and filled with water containing 0.01% surfactant are placed in the layer houses. When the mites enter the jar they are drowned by the water and surfactant and thus cannot communicate with each other. The number of mites in those traps are more representative of the mites actually occurring at that place.

Most monitoring methods or tools are not validated, only tested under practical circumstances. Recently, the AviVet trap, which uses the principle of corrugated cardboard within a barcode-labeled Tylene tube (for identification), has been validated (50). The process of collecting mites is similar to that described above for the regular cardboard traps, but the mites are weighed instead of counted. The validation process indicated that the weight correlates for 99.6% with the counted number of all stages, under the following regression line: $Y = 58.8 + 9.56x$, where Y = total number of mites in the AviVet trap and x = the weight of all mites in the AviVet trap. In addition, the trap was proven to be *D. gallinae*- specific (50).

As all of the above mentioned monitoring techniques are quite labor intensive, an 'automated mite counter' has been developed and validated (23, 49). This counter is placed under the perch. Mites enter the counter through a hole in the lid (again mimicking a hiding space), and are detected and counted by a sensor device. When detected, the mites are removed by air suction into a filter. The mites stay in this filter until removal, which should be done weekly to prevent blockage of the system (23). Validation confirmed the efficacy of the automated mite counter, providing a good estimate of the number of mites, even at low infestation levels. Moreover, the counter seems to be species specific, only counting *D. gallinae* and no other mite species (23). The device is currently commercialized for use in laying facilities as MiteAlert® (49). In this commercial counter the mites are not collected in the filter but blown out by the pump back into the hen house.

Not all of the monitoring methods mentioned above are suitable for routinely sampling by the farmers themselves. Some studies have revealed that farmers favor methods that are not too labor intensive, like the Rickstick method (54), the Simplified Passive Tape (SPT) trap (61), the AviVet trap (50) and the Velcro trap (58) in which a section of Velcro wrapped around the perch

provides a hiding place for mites (41, 71). The automated mite counter (23) is also one of the preferred methods of farmers as it is a practical tool for monitoring with minimal workload.

Location, Frequency, and Duration

The location of the mite traps in the layer houses is crucial for correct monitoring. They should not be placed in mite aggregates, but in passages frequently used by the mites. Near the perches or at cross-points are well-suited localities (50). Traps in nest boxes are less accurate, probably because hens spend less time there, certainly not at night when the mites become active (56). To follow-up the mite population over time in the same poultry house, traps should be placed at exactly the same site on each occasion. Therefore, a detailed map of the trap locations in the layer houses is very useful (50). The number of mites counted with the traps may not be representative of the actual number of mites in the layer houses, but they allow actions to be taken following a change in population size.

A recent project (41) analyzed a large dataset of mite monitoring data to identify the ideal number of traps and trap distribution for obtaining a good insight of the true infestation level. The results revealed that the more traps are placed, the better the insight into the actual infestation level and a minimum of 12 traps per house should be placed. Further, the distribution of mites between and within houses and between different flocks proved to be very variable and thus unpredictable. Therefore, an even distribution of traps over the houses should be applied.

Concerning the frequency of monitoring, again the more frequently monitoring is performed, the better insight is acquired into the population dynamics (72). However, in practice, a compromise should be found with the workload of the monitoring for the farmer. Recommendations by the COST action "COREMI" (FA1404), advise routinely 2-weekly monitoring during commercial production, and weekly in the framework of an experimental design. Although monitoring weekly or 2-weekly, all traps using corrugated cardboard should only be placed in the layer house for about 3 days before removal, as those traps reach a peak of collecting capacity on day 2, and traps that are placed for a longer period are often more damaged by the hens ((65)). Some traps like the Semi Attractive Traps (SAT) (48) and the Rick Stick method (54), can remain for 2 weeks in the layer houses.

The monitoring of mites in layer houses can still be fine-tuned. Quantitative monitoring methods seem more informative than scoring techniques (i.e., no mites- very many mites) (73). However, mites are spread heterogeneously and often unpredictably in a layer house, making it virtually impossible with currently available methods to assess the actual infestation at time "t" with traps. A very high number of traps would be needed to approximate the general infestation level in the house. Also, as mites attract each other to the traps, the number of mites in most traps do not correspond accurately with the number of mites effectively present at the place of the trap (see above). Moreover, comparing actual numbers of mites between houses is difficult as they depend on a large range of different factors (23). Temporal evolution of the population growth within a layer house is thus more relevant (23, 48). The automated mite counter (23) is a method that works with population growth instead of actual numbers.

Actually, the use of simple binary traps might be promising to get relevant information. Chiron et al. (48) already illustrated a very high correlation between a more exhaustive notation of mite aggregates (e.g., four-state scoring) and SPT binary scores. Binary scores are much less labor intensive than multiple-state scoring, and certainly than counting the actual number of mites. Therefore, it would in practice allow the assessment of more traps. As population growth is more relevant to assess the problem (48), and is also used in studies to determine a threshold (see below), the use of a relatively high number of easy-assessable binary methods might the appropriate methodology for use within an IPM strategy.

TREATMENT DECISION BASED ON MONITORING AND THRESHOLDS (STEP 3)

Preventive actions alone are often not sufficient to fully control the pest, and curative means often need to be implemented. Even then, complete eradication of *D. gallinae* is virtually impossible, and control measures should instead be aimed to keep the infestation under a so-called economic threshold, to avoid negative effects on the hens, humans and production. A critical point in an IPM strategy is timing actions (e.g., altering preventative measures or adding treatments) to prevent the increasing pest population from causing damage. By using this "action threshold," treatment/action is not performed too soon and too much, avoiding negative effects on the environment, redundant costs, and resistance emergence. Treatment is also not performed too late so efficient control is still possible.

Unfortunately, such general thresholds are not yet available for controlling *D. gallinae.* This lack of thresholds largely hampers the development of generally applicable IPM strategies for layer houses, and further research is urgently needed. Several monitoring techniques or treatments (see further) provide their own thresholds where they advise treatment is necessary, though these are not scientifically proven.

The difficulty in determining thresholds lies partly in the complexity of determining the actual population size and the multitude of influencing variables (see above). In addition, although production losses due to *D. gallinae* infestations have been evidenced (16), the exact relationships between infestations and bird health/economic impact are insufficiently known. Also here, the many influencing factors hamper the determination of economic thresholds.

A first promising study toward a general critical action threshold was based on concomitant SAT and SPT trapping (48), and working with the temporal evolution of the trapped *D. gallinae* population size. This method is based on Verhulst's mathematical model, which describes the temporal dynamics of populations of living organisms in three successive phases (latency phase, exponential growth phase, equilibrium phase). To allow farmers to treat before the population growth phase,

they proposed (1°) characterize the temporal dynamics of mites by monitoring from the beginning of the flock over several months in order to capture the latency phase and the growth phase by trapping both with a fairly accurate tool and with an any easy-to-use tool in parallel (here SAT and SPT resp.); (2°) identify the moment of maximum acceleration by fitting the Verhulst model to the most accurate data (using a solver tool); (3°) position this key moment on the data obtained concomitantly with the easy-to-use tool and see whether there's a recurring change before he does. Chiron et al. (48) observed in three commercial farms that when for three successive monitoring moments >20% of the placed SPT contained mites, this key moment was approached. Such an SPT-based threshold can easily be applied by the farmer himself. This opens up interesting perspectives for defining a critical threshold that can be associated with monitoring by any means (SPT, Ricksticks, Velcro traps, automated counter, Avivet...). Research on this threshold is still ongoing and it needs further refinement before it can be used with confidence. Although the SPT-based threshold prevents the mite population to start growing exponentially, implicitly avoiding the point of economic damage, it does not explicitly take this economic impact into account.

Another prospect toward treatment advice is the dynamic adaptive model (DAP) (72). The DAP model can predict the population dynamics of *D. gallinae* in a layer house based on monitoring data of the current flock population, temperature data and treatment dates. The model deals with variation in population dynamics by including flock-specific parameters. The model could, however, still be improved by e.g., including more parameters like flock age or husbandry measures. Further research on the DAP model (41, 72) was executed at three different laying hen houses: organic aviary, aviary with winter garden and aviary without winter garden. This research resulted in a practical applicable model forecasting the mite population growth. At the same time, an economic model and an advice algorithm was tested at these farms in order to determine the optimum interval between two treatments in order to keep the population under a certain growth level and to determine if an extra treatment is cost-effective by getting lower numbers of mites and therefore better feed conversion. The threshold and moment when exceeding the threshold was different for all layer houses as e.g., cost price of the eggs, treatment costs and treatment efficacy was different for all layer houses. This illustrates the difficulty of determining general applicable action and economic thresholds.

NON-CHEMICAL TREATMENT- METHODS (STEP 4)

In IPM, the use of chemical (synthetic) control strategies is as much as possible avoided in order to reduce negative effects on human and animal health and on the environment. During the last decades, a lot of effort has been, and is still being, devoted to investigating alternative control measures. The current state-of-the art of alternative control mechanisms against *D. gallinae* is listed below and summarized in **Table 4**.

Although the non-chemical treatments are here listed under step 4, meaning curatively after the mite population has exceeded a threshold, they can within IPM strategies, also be used preventively (step 1) to suppress the growth of the red mite population.

Plant-Derived Products

Plant-derived products have promising potential as alternative non-chemical methods against *D. gallinae*. They can have acaricidal, toxic activity, but also repellent or attractive effects on *D. gallinae* (75, 86, 87). The repellent and attractive effects have much potential in combination with other treatments and are therefore discussed in detail in the section 'treatment combinations'. As plant-based products mostly have low toxic effects on mammals, and are said to have a short environmental persistence, they could have a rather low impact on the environment (74, 75, 77). To act as a repellent, even lower dosages are required then to act as a toxicant (88). These characteristics make plant-derived products very suitable for use in IPM strategies.

Several studies demonstrated that the efficacy of essential oils against *D. gallinae* is mainly attributed to effects of their volatile components (75, 89–91), indicating that they probably have a neurotoxic effect rather than a mechanical one, which indeed has been demonstrated by López and Pascual-Villalobos (92).

The fact that essential oils mainly act through their vapor phase is on the one hand an advantage, as in that way the mites in hiding places can also be reached. On the other hand, the volatile nature probably is the cause of the rather short effect of many oils (88). Another major limitation of the use of plant essential oils is the lack of standardization in formulation, with different batches having differences in chemical composition, which can result in inconsistencies in acaricidal efficacy (75, 88). This problem might be overcome by isolating the active compound of the essential oils, of which eugenol currently seems to be promising, showing the highest toxic effect against *D. gallinae* in several studies (91, 93). In the latter, eugenol also showed to have a repellent effect, changing to an attractive effect over time. Additional research on these active compounds is still necessary.

Further, the efficacy of the majority of these plant-based products has only been demonstrated at lab scale, while in the field, efficacy might be affected by environmental factors like humidity, dust and other pesticides used (91, 94). Also, although some essential oils do not show any negative effect on hens (e.g., thyme), others (e.g., pennyroyal) appear to have an impact on chicken health and egg production (76). Finally, given their short residual toxic effect, plant products may not be suitable as a stand-alone treatment against *D. gallinae*. As such, it has been suggested to combine plant-derived acaricides with other treatments with longer-term effects for a more efficient control of *D. gallinae* (94). Plant-based acaricides are meant to be used curatively, thus ideally after passing an action threshold.

Some plant-based acaricides are commercially available (although not allowed in all countries) for use in layer houses.

TABLE 4 | Overview of main non-chemical treatments.

Treatment	Mode of action	+	−	P/C	Comm.	References
Plant-derived products	Acaricidal, toxic	Short environmental persistence	Short effect	C	X	(74–76)
	Repellent	Potential in attract-and-kill	Lack of standardization	P	X	(77)
Vaccines	Boost immunity	-Low risk for resistance -No workload during production	Further research needed for commercialization	P		(78)
Biological control						
Predatory mites	Prey on PRM	No negative effect on environment (natural enemies)	Also affected by other treatments (silica, acaricides,..)	P(/C)	X	(79, 80)
Entomopathogenic fungi	Penetrate host	Potential in traps	Suboptimal conditions in layer houses	C	X	(81)
Nematodes + endosymbionts			Much research still needed			(82, 83)
Physical control						
Inert dusts (on system)	Dessication of PRM	-Resistance less likely (mainly physical mode of action)	-Health hazards (esp. crystalline) -Variability in effectiveness	P/C	X	(84, 85)
Q perch	Electrify PRM	-No harm to hens -Resistance less likely	Expensive, change in infrastructure	P	X	(62)

Treatments non-allowed in EU (light regime, oils) are not included. Main advantages (+) and disadvantages (–) are listed, as well as their use (P, preventively; C, curatively) and whether they are commercially available (Comm.). For each treatment, main references are given. For further details and other references: see text.

An example is MiteStop® (Alpha-Biocare GmbH), a product based on neem seed extract (*Azadirachta indica)*, which has proven its efficacy against *D. gallinae in vitro* as well as in field conditions (95–97).

Vaccines

With vaccination of hens, there is a low risk of environmental contamination, and the risk for resistance emergence in mites is highly unlikely (98). As such, vaccines have much potential to be incorporated in IPM for *D. gallinae*, principally as a method of prophylaxis as mass administration by injection is not possible once the hens have entered the layer house. Nevertheless, once suitable vaccine antigens have been identified, methods to boost immunity or to administer therapeutic vaccines through e.g., the drinking water during the laying cycle should be explored.

During an infestation, some antigens of the parasite are continuously exposed to the host. These are known as "exposed antigens." In the case of *D. gallinae*, however, the natural immune reaction of the hens against these "exposed antigens" does not seem to be effective for controlling the mites (98, 99). Therefore, the focus for developing vaccines against *D. gallinae* lies on the use of "concealed antigens," i.e., antigens that are not exposed to the host during blood-sucking, like some proteins associated with the midgut of *D. gallinae* (100). This strategy, of using a concealed midgut-related antigen, has previously led to the development of an effective vaccine, marketed as TickGARD, against the cattle tick *Rhipicephalus microplus* based on the BM86 protein (98).

For *D. gallinae* the development of vaccines, including the search for candidate antigens, is still an ongoing process, which has gained momentum since the upsurge of genomics and the recent publication of transcriptomes and the genome of *D. gallinae* (101, 102). Several studies have demonstrated the potential of both native (autogenous) and recombinant antigens for vaccination against *D. gallinae* (78, 98, 99, 103). In an autogenous vaccine, mites for antigen extraction are used from the same premises for which the vaccine will be used. Although authorization for the production and use of these kinds of vaccines may be more easily obtained than for defined vaccines produced on a commercial scale, producing autogenous vaccines is very labor intensive, as mites from every premises that will be treated need to be sampled to make a farm-specific vaccine. Furthermore, in autogenous vaccines, the effective antigen(s) are not well-defined and, as such, poorly quantifiable. Therefore, the efficacy of autogenous vaccines cannot be quantified and may vary between batches. In spite of these difficulties, in a recent field evaluation of both an autogenous vaccine and a prototype recombinant vaccine (78), the autogenous vaccine led to a reduction of 78% of the mite population, while the recombinant vaccine did not show a significant difference in mite numbers compared to the control group. While an autogenous vaccine is therefore currently a possibility for use against *D. gallinae* on a small scale, developing an optimal, standardized recombinant sub-unit vaccine with prolonged efficacy to avoid the need for boosting is the long-term goal (78).

Biological Control

The use of natural enemies against *D. gallinae* is well-framed within IPM strategies, and already commonly applied in horticulture. By using biological control measures, the impact on the environment is minimal and the risk of resistance emergence is avoided.

Predatory Mites

Naturally-occurring enemies can be of great benefit to control pest species. For *D. gallinae*, being an ectoparasite, using enemies that share his living environment is promising (80, 104). The natural communities of enemies of *D. gallinae* occurring in the layer house should be valued, as these already can (partly) control the poultry red mites. Some natural enemies are also artificially reared and commercialized, to mass release them for controlling pest species. This methodology is certainly effective in enclosed systems where the natural enemies are confined to the release site (21). As such, this strategy is already widely applied in greenhouses (105). Candidate predators for commercialization (mass releases) to control *D. gallinae* have been identified by searching for natural occurring predator mites in poultry houses and wild bird nests. *Androlaelaps casalis, Cheyletus eruditus, Hypoaspis aculeifer,* and *Stratiolaelaps scimitus* (previously *H. miles*) are identified as genuine predators of *D. gallinae* (79). As these species naturally occur in layer houses, the risk of a substantial impact on non-target biodiversity is limited, although it remains to be tested. These predators are not *D. gallinae*-specific, also predating on other arthropods and even among predators (80).

Hypoapsis species have a very high predation capacity against *D. gallinae,* higher than that of *A. casalis,* but they appear to be less mobile (106). In addition, for *Hypoapsis* spp., it seems to be impossible to obtain an established population in poultry houses, as specimens were never retrieved a week after release (Koppert Biological Systems).

At least in Europe, currently mainly *A. casalis* and *C. eruditus* are introduced as predators in commercial layer houses, under their respective commercial names ANDROLIS® and TAURRUS® (Koppert Biological Systems, the Netherlands). ANDROLIS® and TAURRUS® can be used complementarily. ANDROLIS mainly feeds on juvenile stages of *D. gallinae* and is a highly mobile and active hunter, while TAURRUS preys on all stages of *D. gallinae* and is a wait-and-sit predator, with a rather slow dispersion. ANDROLIS prefers more humid microhabitats, while TAURRUS prefers drier places (nests, perches,..). Predatory mites are usually released preventively, with the protocol of numbers, frequency and locations of releases depending on the number of hens and housing system. When the populations of *D. gallinae* become too high nevertheless, extra releases of both species can also be done curatively.

The use of predatory mites are promising as a part of a combination of treatments within the IPM strategy, although it should be taken into account that other treatments for *D. gallinae*, like synthetic chemical, plant-based acaricides, or inert substances, also can have deleterious effects on the (natural) predators of *D. gallinae* (80).

Entomopathogenic Fungi, Nematodes, and Bacterial Endosymbionts

There is potential for entomopathogenic fungi, nematodes and bacterial endosymbionts as non-chemical control measures against *D. gallinae.* However, studies on their use against *D. gallinae* in poultry houses are not advanced enough yet, there are still too many hurdles for them to be effectively used in practice, and they are not yet commercially available for use against *D. gallinae*.

Entomopathogenic fungi are frequently used, worldwide but mainly in Latin America, to control pests in crops (21, 81). Fungi infect insects and mites with their spores that adhere to the hosts' cuticle, germinate, and penetrate and spread into the hosts' body (81). Studies under lab-scale conditions have shown that *D. gallinae* is specifically susceptible to *Beauveria bassiana, Metarhizium anisopliae, Trichoderma album,* and *Paecilomyces fumosoroseas* (107–112). However, some experiments in semi-commercial conditions show these fungi are unsatisfactory as a control mechanism against *D. gallinae* (113). In addition, humidity levels in poultry houses are too low to ensure fungal transmission (21); and the effectiveness of these fungi also highly depend on the fungal strain (110). Further, as these fungi are not selective for *D. gallinae* only, they may negatively affect the environment, leading to an environmental disequilibrium (100). However, they are harmless for poultry, eggs and humans (112). These fungi seem to have most potential for being used within traps placed in the poultry house (81), for example within an attract-and-kill strategy (see below). Indeed, a recent study of Nascimento et al. (68), demonstrated the successful use of *Beauveria bassania* in autoinoculation devices (traps with corrugated cardboard or loofah sponge) to control *D. gallinae* in both lab conditions and poultry houses. With this mode of application, problems with non-optimal environmental conditions in poultry houses for the fungi are overcome.

For nematodes, no field experiments on their efficacy against *D. gallinae* have been carried out yet, but using them for controlling flies and beetles in poultry houses has been ineffective, despite positive results in lab-conditions (82). Both fungi and nematodes require particular environmental conditions, such as high humidity levels and free water, making it very hard to use them in practice in poultry houses (8, 21).

Targeting endosymbionts that are vital for mite reproduction and growth is another pathway that is being investigated for control of *D. gallinae*. Although some endosymbionts have been identified in *D. gallinae* with DNA sequencing (83), further research is certainly necessary before it can be used in practice as a treatment.

Physical Control Mechanisms

Hygiene measures, cleaning and disinfection can also be regarded as physical control and these have been discussed in the "prevention" section. Also heat treatment, which can only be applied during the empty period, is discussed in that section.

Light Regime

Studies have shown that mite populations can be affected by disrupting light-dark cycles. Host-searching activity of *D. gallinae*

starts during the dark period, with the highest activity 5–11 h after darkness, so when this dark period is interrupted, their host-seeking activity will decrease and their natural feeding cycle will be disrupted (114, 115). Indeed, the reduction of numbers of *D. gallinae* by short-cycle intermittent light/dark periods has been illustrated by Zoons (116) and Stafford et al. (117), and prolonged darkness compared to standard light regimes resulted in increased number of mites (115). However, disturbing the dark-light rhythm will also largely affect hens, and EU legislation requires a statutory dark period of 8 h (1999/74/EC), so this technique cannot be applied as such in Europe.

Inert Dusts

Inert dusts, or silica-based products, contain *silicon* dioxide as the active biocidal substance, which is, at the time of writing, one of the few permitted biocidal products allowed for treatment of *D. gallinae*. Both synthetic and natural silicas exist. Synthetic products contain only amorphous silicon dioxide; natural products are mainly based on diatomaceous earth and contain a small amount (<1%) of crystalline silicon dioxide. In several regions, only natural silicas are allowed for use at organic farms. However, the amorphous form is considered relatively healthy, while the crystalline form in natural silicas is more harmful to environmental, animal and human health (85, 118). Synthetic silicas are thus actually not truly non-chemical treatments. However, their mode of action against *D. gallinae* is said to be completely mechanical (not poisonous), drying out the epicuticle of the exoskeleton of the poultry red mites through the absorbent character of the silicon dioxide particles, leading to desiccation of the mites (84, 119). Furthermore, resistance against this physical mode of action is less likely to develop than to single-target molecules ((85)).

Many of these silica-based products are commercially available and widely used in Europe. Since dusts consist of fine particles, they can pose hazardous effects to the respiratory tracts of humans. Therefore, a shift is noticeable toward the use of liquid silica-based products, to reduce the deleterious effects of airborne silica (30). However, these effects are not completely eliminated with the liquid application of silica, as some particles become airborne when the fluid has dried, and are further dispersed by hens. *In vitro*, diatomaceous earth seems more effective than synthetic silica (30), though the latter seems to have a longer effect in the field in its liquid form compared to diatomaceous earth (85, 120). The effectiveness against *D. gallinae* has been proven to vary between different silica-based products (especially amorphous silicas) due to variation in absorption capacity of particles, chemical composition, particle size, and specific surface (85). In addition, the effectiveness of inert dusts greatly diminishes at humidity levels of >85% (which can easily be reached in layer houses) (30, 84). Inert dusts are often used preventively in-between flocks, though repeated applications during production are often necessary (84). Even with repeated treatments, silica is often not sufficient as a stand-alone product to control infestations of *D. gallinae*. It is observed that the effect of silica declines after repeated treatments, which could be related to flock age or to the accumulation of dust and debris in laying hen houses over time, hampering the efficacy of silica (72). The latter could be avoided by mechanical cleaning of the surfaces prior to silica application, which has indeed proven to increase the efficacy of silica (120).

Oils

in vitro studies illustrated the effective use of the spraying of mineral oils, diesel oil, petroleum, and plant oils (rapeseed and concentrated orange oil) for poultry red mite control (30). These oils have mainly a physical action by obscuring the stigmata, preventing normal breathing of the mite (106). With diesel oil, there is an associated risk of egg contamination (30). A large disadvantage of oils, is that the greasy substance can stain the eggs, affect the functioning of parts of the system like the egg belt, and they are also rather difficult to remove by cleaning. Further, if not applied everywhere, mites can just avoid the oil spots. Different oils have been used by farmers for years, often successfully controlling mite infestations. However, these oils are not currently permitted for use as a treatment against *D. gallinae,* as there is no biocide registration for any of such products.

Q Perch

The Q perch® (Vencomatic, Netherlands) is a perch where two electric wires and insulators are installed under the cap of the mushroom-shaped perch. Due to the design, the hens cannot be harmed. A small electrical current runs through the wires, killing the mites on their way from their hiding place to the hens nightly resting place (62). The efficacy of the Q-perch is not demonstrated in scientific literature, and installing a Q-perch is rather expensive and a pervasive change of the infrastructure. As it needs to be installed in the layer house, it is used as a preventive measure to control mite population growth, not as a curative measure.

Treatment Combinations

As researchers are becoming more aware that current control mechanisms on their own are not sufficient for controlling *D. gallinae* in layer houses, studies are being performed examining the added value of combining multiple treatments as some can work additively or even synergistically. Sparagano et al. (22) proposed a predicted compatibility matrix of existing and emerging control strategies (their Table 1). However, as only few combinations have actually been scientifically tested, that matrix should be mainly seen as a prediction of compatibilities based on the mode of actions of each treatment separately.

One of the few combinations that has been scientifically demonstrated to have beneficial effects is the combination of the fungus *Beauveria bassania* and inert substances (desiccant dusts) (121). Although this study only comprised laboratory experiments, and the combination still needs to be tested on the field, it illustrates the potential added value of combining treatments.

Two consecutive projects demonstrated the potential of combining predatory mites (*A. casalis* and *C. eruditis*) with two acaricides (milbemectin and amitraz) using impregnated cardboard traps. The test in actual layer houses (cage and aviary systems) illustrated that the combinations had a better efficacy than all the treatments separately. However, the combinations

were still not sufficient to control high *D. gallinae* infestations. "Triple" treatments, where plant-based products were also added, had higher efficacy [(45, 69, 106)].

Like some plant insect pests, *D. gallinae* must find its host at a distance since it does not live on it. *Dermanyssus gallinae* is known to be attracted to temperature gradients and to CO_2 puffs, which are features of homeothermal vertebrates that can be detected at a distance, but are not host-specific. Interestingly, CO_2 is attractive in the dark but induces the mite to freeze in the light, a behavior which apparently allows *D. gallinae* to avoid being eaten by its host (122). *Dermanyssus gallinae* is also attracted by host-specific pheromones, emitted by the hens (123). A cocktail of five volatile compounds among the dozens of compounds naturally emitted by hens has been patented for its attraction demonstrated under controlled conditions in the laboratory (124). Besides host-related attractants, *D. gallinae* is also attracted by aggregation pheromones of congeners, causing clustering together (70, 125). Though the pheromone has not been fully characterized chemically, a series of compounds isolated from the odor of freshly fed *D. gallinae* has been patented as attractants (126).

Interfering in the sensory interactions between *D. gallinae* and its host or between mites among each other are promising avenues to progress IPM, and such approaches may also have minimal environmental impact. The automated mite counter (23) was designed to attract mites as it provides a heat source. Some plant-derived products also act on the sensors of *D. gallinae* as they have a repellent or attractive effect on *D. gallinae* (see above). Attractants and repellents have much potential for use in certain combinations.

The so-called "attract-and-kill" and/or "push-pull" techniques have an interesting potential for a mite that does not live on the host. The principle of the attract-and-kill (or lure-and-kill) technique, widely deployed in crops [e.g., (127)], consists in diverting the pest from its target (host/congeners) by competing odors to specific sites equipped to kill it by various means like acaricides or entomopathogenic fungi. In the case of *D. gallinae,* mite traps (as used for monitoring) impregnated with eradicating treatments are used [e.g., (67)]. Within an IPM strategy, non-chemical killing agents are preferred (24). By using pesticides only on traps, the total amount of pesticide used is also reduced, resulting in less impact on the environment. Further, resistance emergence is slowed down with this methodology as the parasites are exposed to effective doses in a contained environment (70). However, the effective implementation of an attractive substance in such a method has many impediments. The attractant must overrule the natural attractants (host/congeners) interacting with sensory system of *D. gallinae;* an efficient slow-release system for the attractant must thus be developed; and the olfactory attraction must be important in the behavioral choices of *D. gallinae*. The push-pull method combines a repellent activity to push the pest away from its target and attract it to another element. This way the attract-and-kill strategy is actually enhanced by adding a repellent into the process. To our knowledge, however, it has never been used to combat *D. gallinae* to date. Repellents alone can also be used, e.g., by preventing *D. gallinae* to hide into the cracks and crevices, resulting in *D. gallinae* becoming more reachable with (preferably non-chemical) contact products.

Certain plant-based food or drinking water supplements exist for increasing or sustaining the hens health and natural resistance, which also have an effect on the odor of the hen, rendering it less attractive for the mites, and as such working as a repellent (123). This can be beneficial as mites will feed less, and starved mites seem to be more susceptible to acaricides and possibly also to desiccants (128). The potential of combining these supplements with other treatments, i.e., predatory mites and acaricide-impregnated traps, has been demonstrated in field conditions [(45, 69)].

Not all treatments can be used in combination, and some probably have antagonistic effects. Broad-spectrum approaches like silica or heat treatment, e.g., might have adverse effect on the use of natural enemies. Besides combining actual treatments, treatments can easily be combined with simple management actions like cleaning places where hotspots are found with water and soap, to keep the infestation under control, which has proven to be effective (8, 19, 41). The influence of different housing systems and of parameters like temperature and humidity on the efficacy of any treatment (combination) has also been illustrated [e.g., (45)]. Using (or building) systems that are less beneficial for poultry red mites, and limiting the number of potential mite refugia could also help controlling infestations (22). All this illustrates that a holistic approach; integrating biosecurity and prevention measures; appropriate monitoring; attention to different conditions in different housing systems; and interactions with environmental conditions, will be indispensable for effectively controlling poultry red mites in layer houses.

USE OF SELECTIVE/SPECIFIC SYNTHETIC PESTICIDES (STEP 5)

In IPM, the use of synthetic chemical treatments is not completely ruled out. The idea is to only use chemical synthetic products as a last resort, when non-chemical synthetic treatments proved not to be sufficient to control the pest species (24). It is important to thoughtfully select the acaricide and to minimize the total quantity of chemical product that is applied, though respecting the advised dosages to avoid resistance emergence in the pest and optimize the chances of success of the treatment. Preferably, selective products that only affect the target species (*D. gallinae* in our case) should be applied. This way, side effects on non-target species are avoided and the impact on animals, humans and the environment in general is reduced. Unfortunately, at the time of writing, none of the chemical synthetic products available are absolutely selective for *D. gallinae,* as they also have toxic effects on other insects and arachnids.

It is very difficult to obtain information on which synthetic chemical acaricide is currently allowed for use against *D. gallinae*, and different regulations exist for products classified as biocides or veterinary medicines. The products allowed in Europe can be found on the database of the European Medicine Agency database (www.ema.europa.eu) for veterinary medicines, and on

the European Chemical Agency database (www.echa.europa.eu) for biocides. These lists are, however, rather user unfriendly, and farmers request more transparency. Furthermore, they are dynamic and can change daily, making it necessary to regularly check for allowed products. Currently, every EU country has its own national admissions, which makes it even more confusing which product is licensed in a specific country. At the time of writing (2020), only the organophasphase phoxim (ByeMite®), spinosad (Elector®), fluralaner (Exzolt®) and some products of silica/diatomaceous earth are allowed in most European countries (although national regulations should be checked). All these products are allowed during production, although with some restrictions.

The organophosphate phoxim (ByeMite®) is licensed as a veterinary medicine against ectoparasites for livestock (including layer hens), but is not allowed for organic farming. Although some studies report high efficacy of ByeMite® in multiple systems after repeated application (129, 130), others report variable results among different regions and different contact duration (96). In addition, resistance emergence against phoxim is already reported in several countries (see below).

Spinosad (Elector®) is a biological acaricide, and acts upon the nervous system of the mite. Spinosad is licensed as a biocide, and is also allowed for organic farming. Tests in actual layer houses, however, illustrated that the effect of Elector® does not last long and that it is not sufficient as stand-alone treatment (106).

Recently, a fluralaner-based product called Exzolt® has been marketed, which is the first systemic acaricide against *D. gallinae* that is administered orally through the drinking water. It is classified and licensed as a veterinary medicine. It is also allowed in organic farming, though with an extended withdrawal period. Fluralaner is an isoxazoline and inhibits the ligand-gated chloride channels, with a high selectivity for the nervous system of mites, thicks and insects (131). Exzolt® has been proven to kill mites very fast, within 4 h in lab conditions (132). Also in field studies, mite reduction almost occurred immediately, and reductions of up to 100% were observed in all tested units (16, 133). The duration of efficacy (of >90%), however, varied greatly among the tested layer farms, ranging from 56 to 238 days (133).

REDUCTION OF PESTICIDE USE (STEP 6)

Most chemical acaricides are applied in the form of a spray or dust, by which not all hiding mites can be properly reached. Further, spraying can cause stress in the hens; leads to an exposure of the hens and workers to the acaricide; can lead to environmental contamination through e.g., the manure used as fertilizer; and increases the risk of residues in hens and the eggs (18, 133). Indeed, studies on European farms revealed the presence of acaricide residues, even of currently unlicensed substances in tissues and organs of hens and in eggs (18, 134, 135). However, current legal limits (MRL), or MRLs in force at the time the product was allowed, were virtually never exceeded. To reduce the impact on the hens and humans, it is thus essential to reduce the amount of chemical acaricides used in the control of *D. gallinae*.

As the fluralaner-based product Exzolt® is administered through the drinking water, the amount of product that accumulates in the immediate environment is limited (133), though there is concern about the environmental impact of residues in the poultry manure if it is to be used as a fertilizer. Another strategy to restrict acaricide exposure to the environment is to use cardboard traps impregnated with acaricides instead of spraying the whole layer house (65, 67). Hanging sufficient mite traps can, however, be labor intensive, certainly in larger houses, making farmers likely to prefer spraying instead. Another alternative to spraying the whole layer house is only treating hotspots. This is successfully applied in horticulture within IPM strategies (41), and can also easily be applied in *D. gallinae* control. Hotspots with higher infestation levels, where biological treatments thus cannot control the pests, are identified with monitoring. At these restricted places, acaricides are applied with special spray devices to eradicate these populations and to avoid them spreading over the whole area. By treating locally, the total amount of pesticides used is reduced and negative impacts on natural enemies are limited (41).

ANTI-RESISTANCE STRATEGIES (STEP 7)

Resistance against certain controlling treatments is the inheritable ability of an individual to survive this treatment. When an individual is resistant, it will not or only be little affected by the treatment. Resistant individuals will have a competitive advantage over non-resistant individuals, and will thus more likely reproduce. This way, the frequency of their genotype will augment from generation to generation in the treated population. As such, there will be natural selection for these resistant traits. After several generations of selection for resistant genotypes, there will be an evolution towards a general field resistance, and thus a large decrease in the effectiveness of that specific treatment (136). Different underlying mechanisms of resistance exist, and understanding them is crucial to optimally apply anti-resistance strategies (137, 138). For *D. gallinae,* resistance against the formerly widely applied pyrethroids is being studied, with Katsavou et al. (139) showing a high frequency and wide geographical distribution of several non-silent mutations in the voltage-gated sodium channel (the target protein of pyrethroids) associated with resistant phenotypes, though further research is needed. Bartley et al. (140) found that a glutathione S-transferase may have important roles in the detoxification of pesticides and thus in metabolic resistance. Roy et al. (141) detected variations in the enzymatic activity of acetylcholinesterase, the target of organophosphates, among PRM populations sampled in 2008–2009, perhaps foreshadowing some of the current treatment failures.

Resistance of *D. gallinae* against currently and formerly frequently-used chemical acaricides like carbamates, pyrethroids, and the organophosphate phoxim (in

ByeMite®), have been widely reported (8, 18, 142). The likelihood of resistance emergence is increased when products are applied in incorrect dosages, or too frequently, which is reinforced by the limited number of allowed chemicals.

Reducing the use of synthetic chemical acaricides maybe reduces the risk of resistance emergence, but does not avoid it, all the more that resistances to non-chemical treatments are generally far less studied (143). When natural products are commonly implemented in the future, *D. gallinae* population may become resistant against these products too. To date, further research on the underlying mechanisms of resistance against non-chemical treatments is certainly necessary.

To warrant the success of IPM for controlling *D. gallinae*, actions to avoid resistance emergence against these natural as well as chemical treatments should be taken into account. First of all, to decrease the risk of resistance emergence, it is vital not to under-dose nor to exceed the recommended frequency of application of a product. Resistance emergence can be delayed by implementing certain treatments only in traps instead of in the whole system (see above). Resistance is also avoided by combining and/or rotating different products with different modes of action (138). Combining different products with different modes of action is one of the main ideas of IPM and has already been discussed in the section "Non-chemical treatments." Also well-applying preventive management actions could highly reduce the needed amount of other or curative treatments, thus avoiding resistance emergence for those treatments.

EVALUATION (STEP 8)

To assess the efficacy of an applied IPM strategy, and to determine whether adaptations are necessary, a good evaluation of the strategy is needed throughout the whole process. This is primarily done by monitoring the *D. gallinae* population continuously to evaluate the effect of the different treatments (preventive or curative, and non-chemical or chemical), and the IPM strategy as a whole. Besides info on the effectiveness of a treatment, frequent monitoring also provides insight into the duration of the effect. The latter is useful information to determine the cost-benefit of a certain strategy At the stage that IPM strategies will be implemented, the balance between efficacy and (time) costs for a strategy needs to be evaluated, also including economic benefits.

It will be virtually impossible to develop general applicable IPM strategies for every farm. Indeed, a large range of varying factors like house temperature (which is also seasonally influenced), humidity levels, husbandry systems and hen breed, between and even within farms can influence the life cycle of the mites and/or the effectiveness of the treatments applied (72). As such, even the economic and thus also action threshold may vary over farms (see above). Therefore, the aim should be to develop dynamic IPM strategies, with different options depending on specific situations. A continuous evaluation of a strategy at a certain farm at a certain time is thus essential.

CONCLUSION AND FUTURE DEVELOPMENTS

The presented overview shows that for every IPM step, elements are available for control of *D. gallinae* in layer houses. This opens new horizons for researchers in the field to develop practical IPM strategies. However, there are still some important shortcomings and knowledge gaps that hamper the delineation of practical IPM strategies for the control of *D. gallinae* in commercial layer farms.

A major knowledge gap lies in the determination of the action and economic threshold to decide at which point additional action is required. A main confounding factor is the large range of varying factors influencing population dynamics and economic consequences (see above).

In essence, most knowledge gaps lead back to an insufficient fundamental knowledge of the biology and behavior of *D. gallinae*, though some literature exists [e.g., (53, 100)]. As mentioned, improved insights into the population dynamics are essential for the thresholds determinations. Also further research on the actual effects of non-chemical treatments on *D. gallinae* is necessary. Few studies have been performed to examine antagonistic or synergistic effects when two or more treatments are combined, and more scientific research is also necessary for assessing the efficacy of preventive measures, and the potential of an attract-and-kill strategy. Further research on the effect of treatments under field conditions is also necessary as very different results are often obtained in the field compared to a laboratory environment.

In addition, IPM focusses on non-chemical treatments, but EU legislation highly limits the development and the registration of green products and feed additives. With the growing concern of the society related to the negative effects of synthetic pesticides, the EU legislation should search for opportunities for an increased introduction of registered green and safe products on the market.

More knowledge on resistance emergence against chemical and biological tactics and behavioral adaptations to certain treatments and conditions is also indispensable for further development of efficient and sustainable IPM strategies.

Finally, chemical pesticides can be part of an IPM strategy though *D. gallinae*-specific (chemical) pesticides are currently not available and should be developed.

Ideally, some clearly-defined schedules of IPM strategies would be developed that farmers simply need to follow and implement. An attempt was made by Dutch farmers, farm advisers and researchers, making a so-called "*Farm plan for control of PRM at layers*" based on all IPM steps (41). As these farmers found out, and as explained above, a single optimal strategy does not exist as various parameters within poultry houses can affect the efficacy of treatments and the population dynamics of *D. gallinae*. Mul et al. (72) set the basis for a house-specific treatment advice algorithm, indicating the most cost effective point in time for a treatment.

This algorithm is based on a house-specific mite population forecasting model (72) and a house-specific economic model. The models and algorithm were tested at three commercial layer houses and were here able to indicate the point in time for a cost effective treatment to return the mite population to a certain level in the layer house. Further fine-tuning and testing of these models would provide useful tools for IPM implementation on farms. The reasons why some farms only have minor problems with *D. gallinae*, while others suffer from large infestations, even despite several control actions are often unclear. Identifying the reason(s) why some layer houses have lower population growths would provide valuable information.

The aim should thus be to develop dynamic IPM strategies, with different options under different circumstances (see "Evaluation"). Also in horticulture, IPM strategies are composed in such a way, with farmers often hiring IPM advisors for a continuous follow-up and counsel regarding the IPM measures and strategy. The current review highlights which options are available within each IPM step for the control of *D. gallinae* in layer houses, and which important knowledge gaps still need to be tackled to develop practical and efficient IPM strategies, with guidance of advisors. Although this review focusses on layer houses, similar approaches can and should be used in breeder and rearing farms where *D. gallinae* infestations also occur and from which infestations can be brought into a layer house. Indeed, for efficient control, the whole egg production chain needs to be taken into account (19). Further, the current review focusses on the control of *D. gallinae* in European layer houses, but the information can similarly be used for farms outside the EU. Ultimately, control of other pest species in layer houses could be integrated in the strategies.

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ED wrote the main text, with input from all co-authors. NS did the final proofreading.

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Conflict of Interest: JW was employed by the company RSK ADAS, Ltd. AV is employed by the company Koppert BV.

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Case Report: Human Dermatitis Linked to *Ornithonyssus bursa* (Dermanyssoidea: Macronyssidae) Infestation in Portugal

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Ornithonyssus bursa (Berlese, 1888), also known as the tropical fowl mite, is a blood-feeding ectoparasite of domestic and wild birds. It is considered a serious pest to poultry in warm and tropical climates and has been reported to attack humans, causing gamasoidosis. Avian-mite dermatitis may be difficult to recognize and misdiagnosed as bites from other arthropods. The present report describes two cases of human dermatitis linked to *O. bursa* infestation. Both cases occurred in an apartment in a residential area in Oeiras, Portugal, where two members of the same family presented with pruritic erythematous skin eruptions disseminated over their body over a period of 4 months. The lesions were located mostly in the pelvic, gluteal, coccygeal, and perineal regions, and also on the neck, wrists and torso. On inspecting the mattresses and the covers of the bedrooms, three mites ∼1 mm long by 0.5 mm wide were found. The three specimens were identified as *O. bursa*. Investigations tracing back the source of infestation, revealed that the mites were carried over from chickens raised 45 km away (Sesimbra, Setúbal) that, after being slaughtered for consumption, had been transported by car to the apartment in Oeiras. The chickens were farmed in an open backyard henhouse that allowed free access to several species of wild birds, including pigeons (*Columba livia*), turtle doves (*Streptopelia decaocto*), and sparrows (*Passer domesticus*). Recent reports suggest that *O. bursa* may be established in Mediterranean countries, increasing the risk of gamasoidosis. This is the first record linking *O. bursa* infestation of chickens with human dermatitis in continental Portugal. More research is needed to assess the extent of poultry infestation and evaluate the possible implications for the poultry industry, as well as for human health in Portugal.

Keywords: *Ornithonyssus bursa*, avian-mite dermatitis, bird-mite dermatitis, gamasoidosis, chickens, poultry, Portugal

INTRODUCTION

The Dermanyssoidea (*Acari, Mesostigmata)* superfamily comprises most of the mite parasites of vertebrates, including haemathophagous species in the Dermanyssidae and Macronyssidae families, some of which are considered zoonotic (1). Among them, non-burrowing avian mites occur such as *Dermanyssus gallinae* (De Geer, 1778; Dermanyssidae) also known as the poultry red mite, and *Ornithonyssus (O.) sylviarum* (Canestrini and Fanzago, 1877) and *O. bursa* (Berlese, 1888; Macronyssidae), known as the northern and tropical fowl mite, respectively. These dermanyssoid bird mite species naturally parasitize both wild nesting birds and domestic fowl and they also are of veterinary and medical concerns. In fact, in the absence of the natural host, these mites may occasionally feed on humans, causing avian mite dermatitis. Cutaneous manifestations are characterized by pruritic, erythematous, or urticarial papules, often presenting a central sting (2). Due to their small size, about 1 mm in length, and the habit to leave the human skin after feeding (3), infestations may go unnoticed and avian mite dermatitis be misdiagnosed as other skin conditions. In Europe, bird mite attacks are reported among people working with poultry (4) but the incidence of gamasoidosis is increasing in urban settings due to the close proximity of nests of synanthropic birds (2, 4). While *D. gallinae* has a worldwide distribution, *O. sylviarum* is mostly linked to temperate regions and *O. bursa* to tropical and subtropical territories, though recent records suggest it may be also established in Southern Europe. To the best of our knowledge, to date no records for *O. bursa* were available for continental Portugal. The aim of the present report is to describe for the first time a case of avian mite dermatitis linked to *O. bursa* infestation of chickens in continental Portugal.

CASE DESCRIPTION

A 51 years old woman contacted the parasitology laboratory of INIAV (Instituto National de Investigação Agrária e Veterinária) for advice on a possible bird mite infestation. The woman reported that she and her 16 years old daughter, who lived in the same apartment, located in the residential area of Oeiras, had been suffering from pruritic erythematous papules of unknown origin, which occurred continuously, with more or less intensity, over a period of 4 months (February-June 2019). The lesions were located primarily in the pelvic, gluteal, coccygeal (**Figure 1**), and perineal regions, under the breast, and to a lesser extent on the neck, wrists and torso. The intense pruritus and consequent scratching of some papules resulted in wide inflamed skin areas. Lesions were particularly intense and painful around the waist. Bothers occurred habitually at night, but were also noticed during daytime, in which case the waistline was the most affected area. Pruritus was described as severe and prolonged in time and exacerbated by increasing temperature, including by contact with hot water, e.g., while showering or washing the dishes. An initial search in the apartment had not revealed any arthropods visible to the naked eye. After some months, in which no source of dermatitis was found, the mother, who had

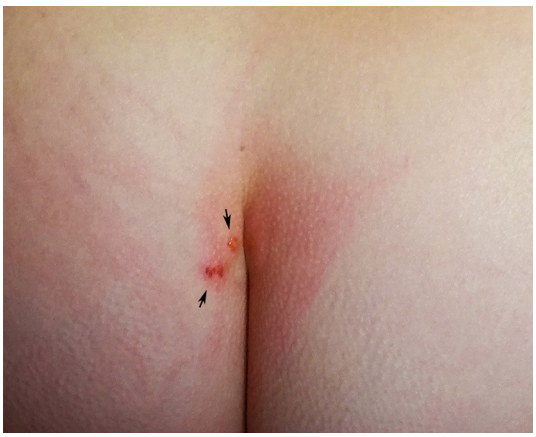

FIGURE 1 | Skin lesions due to *Ornithonyssus bursa* (arrows).

gained experience in the field of veterinary parasitology during her studies, hypothesized that the cutaneous reactions could be caused by bird mites, which, due to their small size could have gone unnoticed. During an interview it was turned out that the affected individuals raised chickens for egg consumption in an open backyard henhouse in their weekend residence in Sesimbra (Setúbal), 45 km away from Oeiras. The owners used to enter the henhouse weekly to collect eggs; direct contact with chickens occurred merely when hens were captured to be slaughtered for consumption. Thereafter, the chickens were usually transported by car to the apartment in Oeiras. No protective clothing was worn during these activities. The henhouse was accessible to several species of wild and synanthropic birds, including pigeons (*Columba livia*), turtle doves (*Streptopelia decaocto*) and sparrows (*Passer domesticus*). Upon questioning, the women also remembered seeing mites crawling on her hands when plucking the chickens. No other sources of mite infestation could be identified, e.g., the presence of active or abandoned bird nests near windows or pets living in the apartment (cats, dogs or birds). Based on the clinical and epidemiological features, it was suspected that dermatitis was caused by avian mites conveyed by chickens from the farm to the apartment. The residents were advised to thoroughly inspect all environments and hiding places suitable for bird mites in the apartment, e.g., beddings, frequently used furniture (desks, tables, couch), cracks and crevices, skirting boards, door and window frames. On inspecting the beddings, the mother retrieved three mites of ∼1 mm on the linens of the bed where she had slept previously. The specimens were sent to INIAV for morphological identification.

Mites were cleared with potassium hydroxide 10%, mounted on microscope slides with Hoyer's medium and identified under a Leica Microsystems™ DM IL LED inverted microscope equipped with phase contrast and integrated modulation contrast (IMC) optics. Photomicrographs were taken with a Leica Microsystems™EC3 digital camera using IMC. The three mites were identified as *O. bursa* females based on the following morphological characteristics: tritosternum present, a posteriorly narrowed genitoventral shield, dorsum of body with relatively

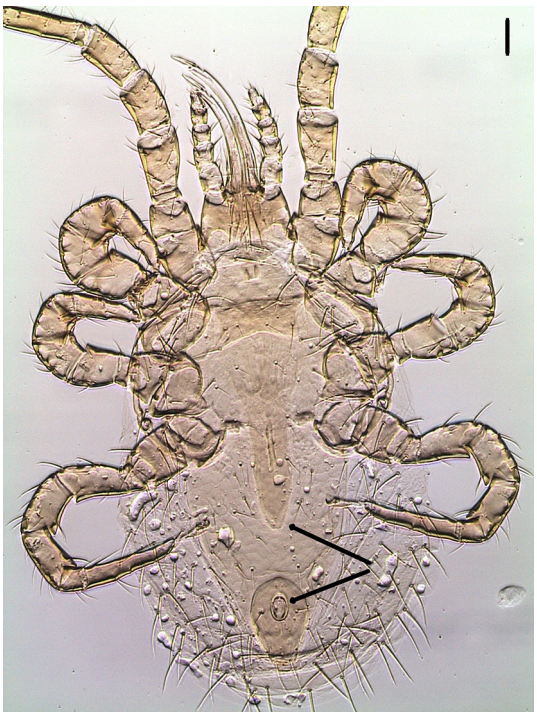

FIGURE 2 | Ventral view of *Ornithonyssus bursa* adult female showing the posteriorly narrowed genitoventral shield (upper arrow) and the teardrop-shaped anal plate with anal opening at the anterior end (lower arrow). Scale bar: 50 μm.

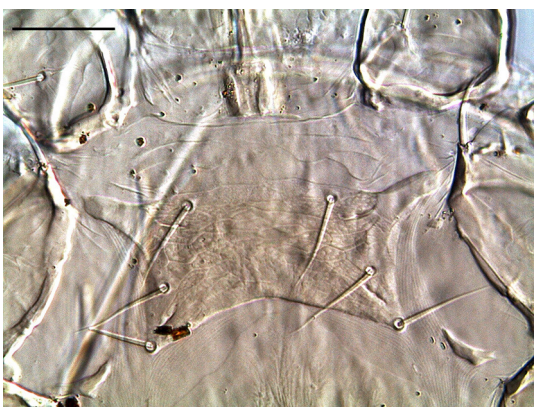

FIGURE 3 | Detail of the sternal shield bearing three pairs of setae. Scale bar: 50 μm.

few setae, elongate, edentate chelicerae, sternal plate wider than long, short, shearlike chelae with well-developed, distinct fixed and movable digits, a teardrop shaped anal plate with anal opening at the anterior end (**Figure 2**), single holodorsal shield gradually decreasing in width posteriorly; a j6 setal pair shorter, not reaching the bases of following pair and the third pair of setae clearly on posterior corners of sternal shield (5–8) (**Figure 3**).

After the identification of *O. bursa*, the woman was asked to search the henhouse in Sesimbra for potential mites, in order to confirm the source of infestation. In addition, she was asked to place cardboard traps 7 x 10 cm in size, commonly used to capture *D. gallinae* (9) near perches and nests. Mites were collected with help of a fine forceps and magnifying glass and stored in 70% alcohol. Traps were frozen at −20° during 24 h to inactivate mites before shipment to INIAV. A total of 17 *O. bursa* and one *D. gallinae* specimens were collected. Most of the *O. bursa* mites were retrieved on perches, which were made of tree-branches, thus providing a perfect place for mites to live and hide near chickens, while the only *D. gallinae* specimens was found in one of the cardboard traps.

Cutaneous manifestations in mother and daughter resolved without medical intervention within 20 days after removal of mites from the apartment. This was achieved by intensive vacuum cleaning, washing of clothes and bedlinen with laundry detergent and hot water (60°C) and thorough daily inspection of mattresses, clothing and bedding. In order to avoid relapses, it was recommended to avoid the contact with infested birds.

DISCUSSION

Gamasoidosis by avian mites is currently an increasing but neglected global problem (4). The causative agents *D. gallinae*, *O. sylvarium* and *O. bursa* are spread worldwide. These mites naturally infect an extensive range of avian hosts, including poultry and several species of wild, pet, and synanthropic birds. Known primarily as pests of poultry, the importance of the three mite species has traditionally been ascribed to different geographical regions and climates. *D. gallinae* is considered the most important ectoparasite for the laying industry, particularly in Europe, where it causes major economic losses to 80% of poultry farms (10). Likewise, *D. gallinae* is highly prevalent in Portugal, with over 90% of layer farms affected (11). *O. sylviarum* occurs throughout the temperate regions of the world, but is primarily important in North America, where it is regarded as the most common and damaging ectoparasite of poultry (12), Brazil (13, 14), China (15), and Australia (16). In Europe, *O. sylviarum* was the most frequent mite in nests of wild birds in Slovakia, Italy and Austria (17–19). It was occasionally also found in poultry (20), pheasants and canaries in France (21), in ornamental chicken breeds in Sweden (22) and, more recently also in pet birds in Portugal (23), but infestation levels are by far not comparable with those of *D. gallinae* (24). The reasons for the different infestation patterns between continents remain unclear (21). *O. bursa*, the least studied of these mites, is considered to be almost entirely restricted to warm and tropical regions. Its presence has been reported in domestic fowl and wild birds in several countries in South America, Africa, Australia, and Asia and islands of the Caribbean Sea, Indian, and Pacific Ocean (6, 25–31). In Europe, its avian hosts include swallows in Denmark (32), rock pigeons and monk parakeets in Spain (33) and backyard chickens in the Madeira Island, Portugal (34).

Human injuries are caused by mites that migrate from abandoned nests of synantrophic birds, like sparrows, starlings, doves and feral pigeons, to residences (2). These bird species are very common in urban areas and usually build their nests on facades of buildings, roofs, windows, chimneys, behind air conditioning units, under eaves, or attics (3, 4, 35). When nests are abandoned, food-seeking mites may intrude residential or occupational settings through ventilation ducts, cracks, and crevices near windows, ceiling and walls. In other instances, avian mites may attack humans working with severely infested poultry or when farmers, workers or visitors are not wearing adequate protective clothing, with *D. gallinae* regarded as an occupational hazard (4, 36). Mite attacks in residential areas occur typically during the night, as opposed to occupational cases, which occur mostly during the day (4). This was also observed in the present situation, though bites were also occasionally noticed during the day, probably due to mites carried inside clothing during working operations. Frequently, patients with bird mite dermatitis present to clinics in late spring and early summer, soon after young birds fledge and adults leave their nests. This was not the case here, because mites were carried over from backyard chickens raised all year round by the owners. Reports on urban cases of avian mite dermatitis have increased in recent years and currently outweigh the reported cases linked to poultry farming (4). *O. sylviarum* and *D. gallinae* are the mites most frequently implicated in gamasoidosis, the latter with over 170 outbreaks registered in Europe (4, 37). Although comparatively less common, human infestations by *O. bursa* have been reported before in urban residential areas of India (38) and Brazil (35, 39–41). In both cases the source of mites was traced to abandoned bird nests. Recently, isolated urban cases of *O. bursa* infestation were reported in an Italian 70-years-old male (Sicily, Palermo) (42) and in a couple (76-years-old, male and 70-years-old, female) in Spain (Girona) (43); the cause of the infestation was attributed to presence of backyard chickens and dove nests in the garden, respectively. Although in both cases mites were only collected on patients and not on birds, the papular dermatitis disappeared after removal of chickens and dove nests, confirming this as the probable source of infestation. In the herein reported case, *O. bursa* was collected and identified in the residence, as well as in the henhouse perches and nests confirming carryover of mites from the infested farm to the apartment.

Despite the increasing number of reports worldwide, gamasoidosis is still a frequently unrecognized ectoparasitosis in humans (2, 3). Diagnosis is challenging, particularly when a direct link to birds cannot be readily established. As humans are unnatural hosts, mites leave shortly after feeding and are only rarely detected on human skin. The small mites are barely visible to the naked eye and may go unnoticed for several months before the causal relationship can be established. In general, gamasoidosis tends to be temporary and self-limiting (44). However, if mites are not found and removed in the environment of the patient, there may be recurrence and exacerbation of the lesions (45). Further, diagnostic errors can occur, because skin manifestations are nonspecific. Therefore, several other ectoparasites need to be considered in the differential diagnosis, including fleas, scabies, pediculosis, baker's itch, *Cheyletiella* bites in pet owners, infestation by Trombiculidae (chiggers), or even bed bug bites (2, 45). Thus, clinical cases can only be precisely diagnosed if ectoparasites are isolated and collected. This case report shows that, although uncommon, poultry farmed in premises outside of the residence area, even if located far away, can act as a source of urban infestation by avian mites. A possible carryover of bird mites from poultry kept elsewhere should therefore be considered in the anamnesis of patients presenting cutaneous manifestations compatible with gamasoidosis.

The present report describes for the first time in Portugal two cases of human dermatitis presumably caused by *O. bursa* and it also confirms the presence of the mite in farmed chickens in continental Europe, also in co-existance with *D. gallinae*. Though a possible carryover of *D. gallinae* into the apartment cannot be completely ruled out, as one specimen was retrieved in the traps placed later in the henhouse, the finding of only *O. bursa* in the bed linens, enabling prolonged skin contact with one of the afflicted individuals, strongly suggests this mite as the cause of the observed dermatitis. Based on previous records in wild and synatrophic birds and human infestation in other Mediterranean countries, the present findings suggest that *O. bursa* can be prevalent in the Mediterranean basin but it could be overlooked or misdiagnosed (38, 39, 42). The typical Mediterranean climate is characterized by warm to hot, dry summers and mild to cool, wet winters; these conditions could be suitable for this tropical mite to develop. Another hypothesis could be a recent introduction of *O. bursa*, either by new wild bird populations or by the importation of fowl or exotic pet bird species, followed by spreading in the Old Continent (42). Irrespective of reasons for actual distribution, the short life cycle of *O. bursa* allows it to quickly build up large populations in poultry farms. Therefore, monitoring of *O. bursa* in wild and domestic avian hosts in Portugal is essential in order to evaluate and mitigate the risk of dissemination to poultry. The increasing number of avian mite attacks to city dwellers, suggest that these infestations may become an emerging public health problem in urban environments. Global warming and ecological changes may favor species range expansion of tropical birds and associated ectoparasites. Likewise, *O. bursa* may expand northward into temperate latitudes increasing the risk for gamasoidosis. More attention to this matter based on a One-Health approach is needed. This supposes a greater awareness of avian dermatitis by physicians/dermatologists and a closer collaboration with veterinarians and entomologists in order to establish a correct diagnosis and treatment, identify the sources of infestation and undertake adequate measures to prevent and control this zoonotic ectoparasite.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HW performed the morphological identification of mites and wrote the report. DA-P and MM provided critical feedback, helped shape the manuscript and contributed to the writing. MM performed a literature review and supervised the work. All authors contributed to the article and approved the submitted version.

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Molecular Detection of *Toxoplasma gondii* and *Neospora caninum* in Domestic Ducks in Hunan Province, China

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Toxoplasma gondii and *Neospora caninum* are protozoan parasites that infect warm-blooded animals, and cause major economic losses in livestock industries worldwide. However, little is known about the genotypes of *T. gondii* and *N. caninum* in domestic ducks in China. Herein, brain samples from 588 domestic ducks from Hunan province in China were examined for the presence of *T. gondii* and *N. caninum*. Polymerase chain reaction (PCR) was used to detect *T. gondii* B1 gene and *N. caninum NC-5* gene. Forty-five DNA samples (7.7%; 95% CI: 5.5–9.9) were positive for B1 gene, and two (0.3%; 95% CI: 0–0.7) were positive for NC-5 gene. The risk factors significantly associated with *T. gondii* infection were age and sex. The 45 samples positive for *T. gondii* were genotyped using multi-locus PCR-RFLP analysis and only one sample was fully genotyped as ToxoDB#9 (Chinese I). These results provide new information about the epidemiology of *T. gondii* and *N. caninum* in ducks in Hunan province in China. The data also highlight the importance of a "One Health" approach to dealing with toxoplasmosis.

Keywords: ***Toxoplasma gondii*, Neospora caninum, domestic ducks, PCR-RFLP, China**

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INTRODUCTION

Toxoplasma gondii and *Neospora caninum* are two important and highly prevalent protozoan parasites (1, 2). Toxoplasmosis, caused by *T. gondii*, is a widespread zoonotic disease causing significantly economic losses in animals and serious public health impacts on humans (3, 4). *T. gondii* in pregnant women may be transmitted to fetus and cause severe neurological sequelae (5, 6). Neosporosis, caused by *N. caninum*, is one of the most important causes of abortion in ruminants, particularly in cattle (7, 8). *N. caninum* is not considered a zoonotic parasite, but low antibody titers to *N. caninum* have been reported in humans (9–11).

Domestic cats and wild felids serve as definitive hosts of *T. gondii*, while dogs and wild canines play the role of definitive hosts of *N. caninum*. Other warm-blooded vertebrate animals (including birds) have been reported as intermediated hosts for both *T. gondii* and *N. caninum* (12–14). Various avian species play an important role in the life cycle of these parasites by serving as intermediate hosts. Avian species can be infected by *T. gondii* and *N. caninum* mainly via ingestion of sporulated oocysts from contaminated environments by feline and canine feces, respectively (15, 16). Domestic ducks serve as a common food source particularly in China. The per capita consumption of duck meat in China was 6.75 kg in 2019. Chinese people often eat undercooked duck meat as roast, spicy or dried. Additionally, duck blood in chili sauce (undercooked food) has recently become popular in many parts of China.

In China, ToxoDB#9 (also named as Chinese I) is the most common genotype in domestic animals, followed by ToxoDB#10 (17, 18). However, limited information is available concerning the molecular prevalence of *T. gondii* in domestic ducks in China. Only one study carried out by Zou et al. (19) showed that genotype ToxoDB#9 was predominant in poultry (including 115 duck meats) in Shandong province of China, indicating that the genetic variation of *T. gondii* in poultry in this province is limited. In addition, low antibody titers to *N. caninum* were found and *N. caninum* DNA was detected in wild waterfowl in Italy, which suggests that wild waterfowl is susceptible to *N. caninum* (20). Nonetheless, domestic ducks as natural intermediate host of *N. caninum* have not been reported.

The aim of the present study was to determine the molecular prevalence, risk factors and genotypes of *T. gondii* and *N. caninum* in domestic ducks intended for human consumption in Hunan province, China. The results provide a baseline for future surveillance and control programs of these parasites in ducks in China.

MATERIALS AND METHODS

Sample Collection

From October 2018 to March 2020, 588 free-range ducks were purchased from food markets in five representative regions of Hunan province, China (**Table 1**). From each food market, ∼5% of the slaughtered ducks were randomly sampled, where brain tissue was collected from each ducks and frozen at −20°C until assayed. Information about the geographic region, season, sex, and age of each duck was gathered.

DNA Extraction and PCR Amplification

Approximately 30 mg was obtained from each brain sample and total genomic DNA was extracted using a commercial kit (Wizard® SV Genomic DNA Purification System, Promega, Madison, USA) according to the manufacturer's directions. A semi-nested PCR was performed to detect *T. gondii* B1 gene (131 bp) as previously described (21). This gene target has been extensively used for detecting *T. gondii* infection in pigs, sheep, chicken, and other animals (22–25). Two primer pairs were used to amplify regions of the B1 gene of *T. gondii*: the outer primers B1-F1: 5′-GGAACTGCATCCGTTCATGAG-3′ and B1-R1: 5′-TCTTTAAAGCGTTCGTGGTC-3′; and inner primers B1-F2: 5′-TGCATAGGTTGCAGTCACTG-3′ and B1-R2: 5′-GGCGACCAATCTGCGAATACACC-3′. PCR product of 191 and 134 bp were obtained from first and second round of PCR reaction, respectively. PCR reactions (25 μl) included 2.5 μl DNA, 12.5 μl commercial premix PPP master mix, 0.1 μl each primers (0.1 mM) and 9.8 μl nuclease-free water. The amplification conditions included a 5 min of initial denaturation at 94°C, followed by 35 cycles of 94°C for 10 s (denaturation), 57°C for 10 s (annealing), 72°C for 30 s (extension), and a final extension step at 72°C for 5 min. The amplification condition for the secondary PCR was identical to the primary PCR, except that the annealing temperature was 63°C (21). Positive (GT1 strain) and negative (ultrapure H_2O) controls were included in each assay.

The *N. caninum* NC-5 gene (328 bp) was amplified using PCR as previously described (26, 27), and by using reaction conditions and primers (Np21: GGGTGTGCGTCCAATCCTGTAAC; NP6: CTCGCCAGTCAACCTACGTCTTCT) described previously (28). The PCR amplification reaction included 3 μl of total DNA, 12.5 μl of commercial premix PPP master mix, 0.1 μl of each PCR prime (0.1 mM) and the remaining 25 μl reaction volume was topped up with nuclease-free water. The amplification conditions included 5 min initial denaturation at 94°C, followed by 40 cycles of amplification (40 s at 94°C, 40 s at 94°C, 40 s at 72°C and a final extension step at 72°C for 10 min. Positive (*N. caninum* NC-1 strain) and negative (ultrapure H_2O) controls were included in each assay.

Each PCR product was examined on agarose gel (1%) electrophoresis to verify that they presented the expected bands of the target genes. The positive PCR products for the NC-5 gene were submitted to the Sangon Biotech Company (Shanghai, China) for DNA sequencing.

Genetic Characterization of *T. gondii*

The B1 gene-positive samples were genotyped at 10 genetic markers (SAG1, SAG2 (5′+3′ SAG2, alter. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico) using the multi-locus PCR-RFLP analysis as previously described (29) (**Table 2**). Eight reference *T. gondii* strains (GT1, PTG, CTG, MAS, TgCgCa1, TgCatBr5, TgCatBr64, and TgRsCr1) were included as controls as reported in previous studies (33, 34). The genotype was determined by comparing its multilocus pattern to the pattern of all genotypes present in ToxoDB (http://toxodb.org/toxo/) (35).

Statistical Analysis

The data were analyzed using SPSS 20.0 (IBM, Chicago, IL, USA). Multivariable mixed-effects logistic regression model was used to determine the relationship between the prevalence of *T. gondii* and various factors related to the ducks examined in the study, including the geographic region, the season of collection, age, and sex. Probability (P) value <0.05 was considered as statistical significance.

RESULTS

In this study, the overall prevalence of *T. gondii* in domestic ducks in Hunan province was 7.7% (95% CI: 5.5–9.9) (45/588). The prevalence of *T. gondii* infection in domestic ducks was 2.6%, 9.2, 9.3, 11.5, and 4.6% in Eastern, Southern, Central, Western, and Northern Hunan, respectively. However, there was no significant statistical difference in domestic ducks from different regions ($P > 0.05$) in Hunan province compared to Western region ($P < 0.05$) (**Table 1**). The prevalence of *T. gondii* infection in different seasons is shown in **Table 1**. The highest prevalence was found in Autumn (11.5%; 95%CI:6.6–16.4), followed by Summer (9.7%; 95%CI: 5.4–14.0) and Spring (2.4%; 95%CI: 0–5.7), and these differences were statistically significant ($P < 0.05$) compared to Winter. The prevalence of *T. gondii* in domestic ducks of 1<years≤2 (8.6%; 95% CI: 6.1–11.1) was higher than ducks of 0<year≤1 (2.2%; 95% CI: 0–5.2) (**Table 1**), and these differences were statistically significant ($P < 0.05$). Logistic

TABLE 1 | Prevalence and risk factors for *Toxoplasma gondii* infection in domestic ducks in Hunan province, China.

Factor	Category	No. tested	No. positive	Prevalence (%) (95% CI)	Adjusted Odds ratio (95% CI)	*P*-value
Region	Eastern	78	2	2.6 (0–6.1)	Reference	
	Southern	153	14	9.2 (4.6–13.8)	3.8 (0.8–17.3)	>0.05
	Central	75	7	9.3 (2.7–15.9)	3.9 (0.8–19.5)	>0.05
	Western	131	15	11.5 (6.0–17.0)	4.9 (1.1–22.1)	<0.05
	Northern	151	7	4.6 (1.3–7.9)	1.8 (0.4–9.1)	>0.05
Season	Spring	84	2	2.4 (0–5.7)	Reference	
	Summer	186	18	9.7 (5.4–14.0)	4. 4 (1.0–19.4)	<0.05
	Autumn	165	19	11.5 (6.6–16.4)	5.3 (1.2–23.5)	<0.05
	Winter	153	6	3.9 (0.8–7.0)	1.7 (0.3–8.5)	>0.05
Age	0<year≤1	89	2	2.2 (0–5.2)	Reference	
	1<years≤2	499	43	8.6 (6.1–11.1)	4.1 (1.0–17.2)	<0.05
Sex	Male	181	6	3.3 (0.7–6.0)	Reference	
	Female	407	39	9.6 (6.7–12.5)	3.1 (1.3–7.4)	<0.01
Total		588	45	7.7 (5.5–9.9)		

regression analysis showed that ducks 1<years≤2 of age (OR: 4.1; 95% CI: 1.0–17.2) had four times higher risk of being positive compared with ducks ≤1 year old. As is shown in **Table 1**, female ducks (9.6%, 95% CI: 6.7–12.5) had a higher prevalence than male ducks (3.3%, 95% CI: 0.7–6.0), and these differences were statistically significant ($P < 0.01$). Logistic regression analysis showed that female ducks (OR: 3.1; 95% CI: 1.3–7.4) had three times higher risk of acquiring *T. gondii* infection compared with male ducks. In the present study, only one brain sample was genotyped at all loci, which was identified as genotype ToxoDB#9 (**Table 2**).

Two (0.3%; 95% CI: 0–0.7) of the 588 examined brain samples were positive for *N. caninum* Nc-5 gene. The sequences of the amplicons of both samples were deposited in GenBank (GenBank accession nos. MW194292 and MW194293). The Nc-5 gene sequences of *N. caninum* had 99% similarity to *N. caninum* sequence published previously (GenBank accession no. KU253799).

DISCUSSION

The prevalence (7.7%) of *T. gondii* in ducks in present study was higher than that reported in doves (*Zenaida macroura*) in American country (1%) (36); pigeon (*Columba livia*) in Iran (6.9%) (37); wild ducks in the Czech Republic (38); and poultry in Shandong (7.37%) (19). However, this prevalence was significantly lower than that detected in starlings (*Sturnus vulgaris*) (12.8%); chickens (*Gallus domesticus*) (15.5%) and sparrows (*Passer domesticus*) (26.5%) in Iran (37) and sparrows (*Passer domesticus*) in Brazil (17.5%) (39). These differences might be related to different avian species or different husbandry practices.

The results showed that ducks 1<years≤2 of age (OR: 4.1; 95% CI: 1.0–17.2) had four times higher risk of being positive compared with ducks ≤1 year-old, indicating that age may be a risk factor for *T. gondii* infection, in agreement with previous studies (40–43). Age is widely considered as a risk factor for high infection rates of *T. gondii* (44, 45). This might be attributed to increased frequency of exposure to the infectious *T. gondii* oocysts or the cumulative effect of the time period during which an animal can be exposed to the parasite (43, 46). The present study also showed that female ducks (OR: 3.1; 95% CI: 1.3–7.4) had three times higher risk of acquiring *T. gondii* infection compared with male ducks, suggesting that female ducks are more susceptible to *T. gondii* than male ducks (47).

In the present study, only one brain sample showed complete genotype at all loci, which was identified as genotype ToxoDB#9 (**Table 2**), which is consistent with that reported in ducks in a previous study in China (19). In addition, the remaining 44 B1-positive samples were amplified at only 3–5 loci, so have limited significance to reveal the level of genetic variation of *T. gondii*. To date, although different genotypes of *T. gondii* have been reported in domestic avian species worldwide (*e.g.,* ToxoDB#2, 9, 10, 26, 53, 114, 225, 227, 278, 281, 282) (19, 48–52), ToxoDB#9 is the prominent genotype in domestic poultry in China, and has been also frequently reported in other animals in China (53). A previous study (19) indicated that only one genotype (ToxoDB#9) was identified from domestic ducks, suggesting that the genetic variation of *T. gondii* may be relatively low in domestic ducks in China. However, further investigations including more domestic duck samples from other provinces of China are required to ascertain the full extent of *T. gondii* genotypes in domestic ducks.

Although previous studies showed that *N. caninum* DNA has been detected in domestic and wild poultry (20, 54), it was not detected in domestic ducks. The present study revealed a low molecular prevalence (0.3%) of *N. caninum* in domestic ducks, which is significantly lower than that reported in wild waterfowl (28.6%) (20) and chickens (4%) (54). Differences in *N. caninum* prevalence are likely attributed to differences in climates, husbandry practices, detection methods, or geographical origins. Our finding provided further evidence that domestic ducks are

TABLE 2 | Genotyping result of *Toxoplasma gondii* infection in domestic ducks in Hunan province, China.

Isolate ID	Host	Tissue	Location	SAG1	5'+3' SAG2	Alternative SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Genotype	References
GT1	Goat		United States	I	I	I	I	III	III	I	I	I	I	I	Reference, Type I, ToxoDB #10	(29)
PTG	Sheep		United States	II/III	II	II	II	II	II	II	II	II	II	II	Reference, Type II, ToxoDB #1	(29)
CTG	Cat		United States	II/III	III	III	III	III	III	III	III	III	III	III	Reference, Type III, ToxoDB #2	(29)
MAS	Human		France	u-1*	I	II	III	III	III	u-1*	I	I	III	I	Reference, ToxoDB #17	(29)
TgCgCa1	Cougar		Canada	I	II	II	III	II	II	II	u-1*	I	u-2*	I	Reference, ToxoDB #66	(30)
TgCatBr5	Cat		Brazil	I	III	III	III	III	III	I	I	I	u-1*	I	Reference, ToxoDB #19	(31)
TgCatBr64	Cat		Brazil	I	I	u-1	III	III	III	u-1	I	III	III	I	Reference, ToxoDB #111	(31)
TgRsCr1	Toucan		Costa Rica	u-1	I	II	III	I	III	u-2	I	I	III	I	Reference, ToxoDB #52	(32)
Sample #105	**Duck**	**Brain**	**Hunan, China**	**u-1**	**II**	**II**	**III**	**III**	**II**	**II**	**III**	**II**	**II**	**I**	**ToxoDB #9**	**Present study**

**u-1 and u-2 represent unique RFLP genotypes, respectively.*

natural intermediate hosts *N. caninum*. Previous studies (55–57) have shown that poultry get infected with *T. gondii* through ingestion of sporulated oocysts from a contaminated soil. So, it is possible that domestic ducks become infected with *N. caninum* via the same route.

Humans become infected with *T. gondii* mainly via ingestion of raw or undercooked meat of infected animals (58). The present and previous (19) studies revealed the presence of *T. gondii* infection in domestic ducks in China, highlighting the potential threat to human health. According to the Ministry of Agriculture and Rural Affairs of China, 9,444,400 metric tons (about 70% of the global total) (59) of duck meat was produced and consumed in China in 2019. Duck meat including roast, spicy and dried duck meat is very popular among most Chinese. More importantly, pregnant women are encouraged to consume duck products (including duck blood) due to cultural habits. The risk of *T. gondii* infection in humans greatly increases by eating undercooked infected meat or meat products obtained from ducks. Therefore, adequate cooking of potentially infected duck meat is the safest way to ensure that tissue cysts are deactivated, thereby preventing infection. The results of the present study should assist the duck meat industry and local regulatory agencies to optimize interventions to improve the safety of duck products.

CONCLUSION

The present study provided new data on the prevalence and risk factors of *T. gondii* infection in domestic ducks in Hunan province, China. To our knowledge, this is the first study focusing on *N. caninum* in domestic ducks in China. Future studies should consider studying histopathological changes and viability assessment of the parasites present in the duck tissues. Our findings provide a baseline for future surveillance and control of these parasites in ducks in China and reaffirm the importance of a "One Health" approach to dealing with toxoplasmosis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: GenBank and accessions MW194292 and MW194293.

ETHICS STATEMENT

The study was approved by the Ethics Committee of Hunan Agricultural University (No. 43321503).

AUTHOR CONTRIBUTIONS

G-HL conceived and designed the study and critically revised the manuscript. Q-YL performed the experiments, analyzed the data, and drafted the manuscript. H-LZ and W-HY helped in the study design. All authors read and approved the final manuscript.

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Who Is *Dermanyssus gallinae*? Genetic Structure of Populations and Critical Synthesis of the Current Knowledge

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Despite the economic and animal welfare importance of the Poultry Red Mite *Dermanyssus gallinae*, its genetic structure has been studied in a scattered way so far. The prophylaxis and control of such a globally distributed ectoparasite can be significantly improved by understanding its genetic population structure (composition in species and intraspecific variants). The present study aims to establish a rigorous framework for characterizing the neutral genetic structure of *D. gallinae* based on a literature review combined with an integrative analysis of the data available in GenBank on population-level nucleotide sequence diversity supplemented by a new dataset. The integrative analysis was conducted on sequence data extracted from GenBank coupled with new sequences of two fragments of the mitochondrial gene encoding Cytochrome Oxidase I (CO1) as well as of an intron of the nuclear gene encoding Tropomyosin (Tpm) from several PRM populations sampled from European poultry farms. Emphasis was placed on using the mitochondrial gene encoding CO1 on which the main universal region of DNA barcoding in animals is located. The species *D. gallinae sensu lato* is a species complex, encompassing at least two cryptic species, i.e., not distinguishable by morphological characters: *D. gallinae sensu stricto* and *D. gallinae* L1. Only *D. gallinae s.s.* has been recorded among the populations sampled in poultry farms worldwide. Current knowledge suggests they are structured in three mitochondrial groups (haplogroups A, B, and C). Haplogroup A is cosmopolitan, and the other two present slightly contrasted distributions (B rather in the northern part of Europe, C most frequently found in the southern part). Recent data indicate that a dynamic geographic expansion of haplogroup C is underway in Europe. Our results also show that NUMT (nuclear mitochondrial DNA) pseudogenes have generated artifactual groups (haplogroups E and F). It is important to exclude these artifact groups from future analyses to avoid confusion. We provide an operational framework that will promote consistency in the analysis of subsequent results using the CO1 fragment and recommendations for future analyses.

Keywords: *Dermanyssus gallinae*, haplogroups, mitochondrial DNA, CO1, tropomyosin, NUMTs, one-health, epidemiology

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INTRODUCTION

A good understanding of the genetic structure of the populations of any parasite is a crucial prerequisite to optimize its prophylaxis and control. Indeed, the development of management tools can be improved if we know whether one or several species are involved in the infestations and if we trace the routes of dissemination of the populations. The tremendous economic, health, and animal welfare importance of the Poultry Red Mite (PRM), *Dermanyssus gallinae* (DeGeer, 1778), coupled with recurrent prophylactic and control failures, highlight the need for knowledge on the genetic structure, and population dynamics of this parasite. However, substantial gaps have been identified by the collaborative work of the COST COREMI FA1404 Action (https://www.cost.eu/actions/FA1404/#tabs|Name:overview) and Discontools.eu (https://www.discontools.eu/database/112-poultry-red-mite.html). Consequently, efforts in improving these areas are needed. Indeed, not only is PRM one of the under-studied parasites of economic importance, but the few published molecular studies of PRM come from the work of research teams belonging to different disciplinary fields (veterinary and medical parasitology and microbiology, pharmacology, evolutionary biology, taxonomy etc.,). They are published in journals from various disciplinary fields, which makes the pooling of results delicate. Four studies published between 2009 and 2011 (1–4) had established the first basis for the characterization of genetic groups within the genus *Dermanyssus* Dugès, 1834, with a focus on *D. gallinae*. Only one was published in a veterinary journal (3). Recently, several studies on the genetic structure of *D. gallinae* in layer farms in different regions of the world, mainly from Europe, have been published. Based on fragments of the gene encoding Cytochrome Oxidase I (CO1), some of them revealed genetic groups whose biological status (species or intraspecific variants) had not been determined. These groups could either be biological species in the sense of Mayr (5), i.e., groups of individuals that may interbreed with each other but not with different populations (groups i), or be intraspecific variants, interfecund with other populations of *D. gallinae s.s.* (groups ii), or constitute illusory groups, resulting from the presence of pseudogenes in the dataset (DNA sequences treated as homologs of others when they are not) (groups iii). The need to establish a standardized framework allowing an optimized and harmonized approach to analyze future molecular data on PRM guides the present study. This will improve our understanding of the problems associated with PRM infestations as it will allow more data to be compared across specific studies.

Let's start by laying the theoretical background for the exploration we propose to carry out. The formation of biological species in the sense of Mayr (5) (groups i) and of intraspecific variants (groups ii) result from the same evolutionary process, speciation, at more or less advanced stages [see (6) and lexical notes in (4)]. The genotypic composition of populations is constantly changing over time due to random events (mutations, genetic drift), and natural selection. The interruption of gene flow by any barrier that prevents cross-breeding between populations (e.g., geographical isolation) inexorably leads from generation to generation to their genotypic differentiation and consequently to phenotypic differences affecting various characteristics (morphology, physiology, behavior, etc.). After a certain time (numerous generations), reproductive incompatibility (a complete lack of reproduction between the individuals or reproduction resulting in infertile progeny) may appear and the entities thus differentiated are no longer able to exchange genes even if the barrier can be broken (i.e., through new contamination routes or movements by hosts): these end up as separate Mayr's species (5). But before complete separation occurs, such a break in barriers can lead to highly differentiated populations exchanging genes again [speciation reversal between incipient species (7)]. Deciding between the two above categories is far from evident since determining whether reproductive incompatibility has occurred between populations is practically impossible. We typically look for clues of disruption within a continuum of variation by bringing together different lines of evidence [molecular phylogenies, morphological analyses, ecological, and behavioral observations, etc., (8)]. Identifying these clues is a significant issue in pest management as considerable differences in pathogenicity, pesticide resistance, vectorial capacity, etc., can be associated with different pest species (or incipient species) as a result of the absence of gene flow between each another for many generations, regardless of whether or not they exhibit detectable morphological differences.

Cryptic species are populations that are reproductively isolated from others (= Mayr's species) but for which no morphologically discriminating characters have been identified (9). Nevertheless, they are likely to differ in various non-morphological characteristics as much as from closely related species that may be morphologically distinguished (9) and that may be present in the same environment. For example, aspergillosis agents were considered multi-resistant to antifungal agents before it was discovered that they were composed of different cryptic species, each with a narrow resistance spectrum (10). The distinction between these morphologically identical variants is clinically needed to treat the induced pathology (10) properly. Cryptic speciation has been widely described, especially in taxa of tiny invertebrates such as mites after molecular tools have been employed, leading to an understanding of a grossly underestimated diversity (11).

In addition, pseudogenes may confuse the information provided by a single region (groups iii): particularly in mitochondrial analyses, where these non-functional variants of the region of interest can be amplified with mitochondrial DNA and aligned as if they were orthologous sequences (12–14). These paralogous sequences result from past incorporation of mitochondrial DNA copies into the nuclear genome and are known as NUMTs for "nuclear mitochondrial DNA". They do not reflect the same evolutionary history as the targeted genomic region and can generate artifact genetic groups in analyses (with percentages of divergences from conspecifics typically greater than intraspecific values). This happens because NUMTs lose their functionality when integrated into the nuclear genome and are therefore freed from selective pressures usually experienced by functioning genes. In addition, they are subjected to different molecular repair mechanisms after being incorporated into the

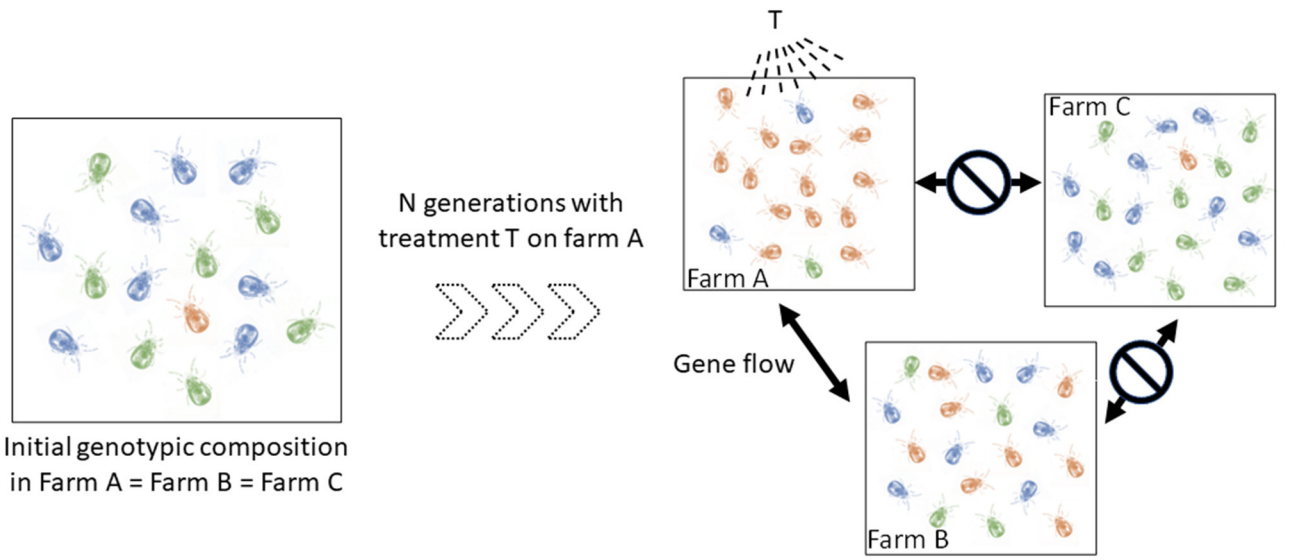

FIGURE 1 | Schematic illustration of the implication of spatio-temporal dynamics in the diffusion of a dominant resistance to antiparasitic agents. Let consider three hypothetical farms, two of which are interconnected (farm A, farm B), i.e., they exchange mites regularly, and one isolated farm (farm C). Left, the blue and green genotypes are sensitive to the T treatment (only sensitive alleles), the orange is resistant to it (i.e. any genotype containing the resistant allele) (to simplify, we start from identical initial genotypic compositions in the three farms, with the resistant genotypes (i.e. any genotype containing the dominant allele) being very infrequent). *Right*, genotypic composition after a certain number of generations of mites having undergone the selective pressure of the T treatment in farm A and having received no treatment in farms B and C. In farm A, mites carrying the resistant allele (orange) were favored by the T treatment and thus transmitted this allele to a large number of offspring. The resistant genotypes thus became the majority. Because of the gene flow between farms A and B and the isolation of farm C, this resistant allele has increased in frequency in farm B and not in farm C. If farmers B and C start applying the T treatment at this time, the former is likely to experience treatment failures very quickly, while the latter will be able to treat effectively for some time.

host nuclear DNA. Distinguishing cryptic species (groups i) within mitochondrial variants from the artifact groups produced by NUMTs (groups iii) is, therefore, crucial to clarify the genetic structure of a parasite and to advance the understanding of its epidemiology.

In addition, a better understanding of the spread routes between populations of mites (= infestation routes between farms) is also central to progress on the knowledge of their epidemiology. Individuals from two connected populations have almost as much chance of reproducing with each other as within one of them. In contrast, individuals in less connected populations have more chance to reproduce with individuals inside their own population. Such a simple reduction of gene flow can produce population differentiation (groups ii) whose effect in terms of clinical management is not negligible. Identifying the direction and location of gene flow amongst infested farms can shed important light on infection events and the origin of infections. Importantly, this information is relevant for making prophylactic recommendations. This also has direct relevance for predicting the evolution and spread of anti-parasitic resistance, should that appear in the mite population. The degree of connection between farms is expected to vary according to a number of parameters closely related to poultry transfer routes, including logistic networks, geographical proximity, and sanitation regimes. To illustrate, if the population in farm A has developed resistance to T treatment and is strongly connected to farm B's and not connected to farm C's, genotypes with resistance to T treatment are more likely to spread to the farm B than to farm C, especially if the resistant allele is dominant (**Figure 1**). Therefore, if farmers B and C start applying the T treatment for the first time on their farm at the same time, farm B is likely to experience treatment failures very soon, while farm C will take much longer to experience this problem. Of course, the kinetics of resistance emergence in farms B and C will also be modulated by the fitness cost of the resistance to T treatment, but the dynamics of the development of resistance to the treatment may be strongly different between these farms. Mapping in time and space the intraspecific variants (possible groups ii) allows the inference of the spread routes of the organisms [e.g., (15, 16)].

Now let us come to the state of the art concerning Poultry Red Mites. To understand epidemiological patterns of *D. gallinae* infestations and its resistance to parasiticides, a correct knowledge of any boundaries between species (groups i: who is who?) is necessary, as well as of epidemiological dynamics (groups ii: who goes where?), which requires the selection of relevant genetic markers (prevent artifacts due to groups iii). Regarding the species composition of the genus *Dermanyssus*, Moss (17, 18) provided during the 20th century the only in-depth taxonomic study of the entire genus *Dermanyssus* using morphology alone, with helpful dichotomous keys to the 18 species described so far. The genus *Dermanyssus* now contains 25 described species and is divided into two subgenera, *Dermanyssus* and *Microdermanyssus* (1). The subgenus *Dermanyssus* is divided into two groups of species: the *hirsutus* group and the *gallinae*

group (17). Here we focus on the *gallinae*-group, which contains the economically important *D. gallinae* along with 14 other species. Moss (18) claimed that diagnostic morphological characters were difficult to define and explicitly urged those seeking to identify mites of the genus *Dermanyssus* based on morphology to be cautious in the use of his key because of the extensive intraspecific and intrapopulation variation in the diagnostic characters that he retained (although after more than 10 years of careful examination). In their studies, Roy et al. (1, 2, 4) have focused on the interspecific delineation of the *gallinae*-group within the *Dermanyssus* subgenus by combining morphological and molecular analyses. The latter two studies (2, 4) questioned the fine lines by considering intra-population variations. Roy et al. (1) confirmed the difficulties of morphological identification, set the interspecific boundaries between widely divergent entities and solved some morphological issues by pointing out segregating (allowing a species diagnosis) and overlapping (blurring the distinction between species) characters within genetic groups defined by phylogenetic analysis of mitochondrial and nuclear sequence alignments. Roy et al. (1, 19) reported several mitochondrial groups as possible species presenting a less deep divergence but did not validate them due to lack of corroboration by more than one line of evidence recommended by DeSalle et al. (8). Roy et al. (2) and Roy and Buronfosse (4) have partly filled this gap and confirmed some interspecific boundaries (cryptic species *D. gallinae* L1) but not all (clade E, isolate JOW). Interestingly, the haplotypic and nucleotidic diversities of both mitochondrial and nuclear regions were found to be substantially higher within *D. gallinae* populations compared to other species of the genus *Dermanyssus*, suggesting recurrent events of hybridization between incipient species in the pest species (1, 4). Regarding the population composition within *D. gallinae*, seven articles deal with the population structure within *D. gallinae* based on mitochondrial DNA sequences with the aim to answer questions related to the management of the parasite in the poultry industry (3, 4, 20–24) whereas, three studies used mitochondrial DNA sequencing to distinguish two strongly differentiated genetic pools within *D. gallinae* in a human pathology context (25–27).

The objective of the present study was to summarize and critically review the knowledge on the mite population and species structure obtained so far using Sanger-sequencing technology in the Moss' *gallinae*-group within the genus *Dermanyssus* with a focus on the species of economic and animal welfare importance, namely *D. gallinae*. This work is based both on a review of the literature and on an integrative analysis of the DNA sequence data associated with the publications, supplemented by the acquisition of new data. In particular, we will take the opportunity to clarify the groupings delineated so far within *D. gallinae* based on CO1 gene by establishing synonymies between them and by spotting the artifact groups generated by pseudogenes. Such critical elucidation - with a didactic approach -, supplemented by the analysis of new molecular data and by recommendations, will help researchers to provide a more efficient standardized handling of molecular tools, able to better answer questions related to the biology and epidemiology of *D. gallinae* and similar organisms.

MATERIALS AND METHODS

Literature Review

The available literature on the taxonomy of the genus *Dermanyssus* and the neutral genetic structure of *D. gallinae* was reviewed by searching research papers through three electronic databases (Scopus, Web of Science and Google Scholar) until December 2020 with no time limits. A review has been realized by highlighting the historical development of the successive discoveries and the unsolved questions.

Sequence Data

The mite sequences included in the studies listed in **Table 1** were collected from the GenBank database (per October 2020) and an unpublished repository (unpublished data, LR). The new molecular data were acquired from mites collected from bird farms (**Table 2**). The mites were individually processed. The DNA extraction from each individual was performed with the REDExtract-N-Amp Kit (Sigma-Aldrich, Saint Louis, MO, USA), following the manufacturer's recommendations after a rough crushing with a pipette cone. One or two of the regions of the CO1 gene defined in **Figure 2** were amplified in all individuals with the primer pairs shown in **Table 3**. To verify that the different primer pairs used in the various studies did not amplify different CO1 sequences in the same individual (NUMTs or heteroplasmia), some individuals were treated with two different primer pairs. To refine the understanding of the genetic structure at the borderline between sister species and intraspecific variants, we also amplified the intron n of the Tropomyosin (Tpm) coding gene in a subsample of these new mites (see **Table 2**) with the primers listed in **Table 3**. PCR was performed with a 11-µL total reaction mix and 2 µL of diluted DNA (up to 20 ng) in a Perkin-Elmer PE 9700 thermal cycler (Perking-Elmer, Waltham, USA). The reagent concentrations were 1 x PCR buffer (Qiagen), 0.04 U/µL Qiagen *Taq* Polymerase, 200 µM dNTPs, and 0.3 µM of each primer, and 3.4 mM of $MgCl_2$ for CO1 and 1.5 for Tpm. DNA amplification was carried out as follows: initial denaturation at 94°C for 5 min, followed by 5 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 48°C (CO1) or 56°C (Tpm), elongation for 1 min at 62°C (CO1) or 72°C (Tpm), 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 52°C (CO1) or 56°C (Tpm), elongation for 1 min at 62°C (CO1) or 72°C (Tpm), and final extension at 62°C (CO1) or 72°C (Tpm), for 10 min. Negative controls (without DNA matrix) were included in all PCR runs and amplification products were verified by 1% agarose gel electrophoresis and Ethidium Bromide staining. Both strands of final PCR products were Sanger sequenced using a commercial sequencing-service (MWG Eurofins Operon sequencing laboratory, Germany). The chromatograms' quality was checked using Chromas v2.6.6 (http://technelysium.com.au/wp/chromas/) and were manually corrected in case of misinterpreted peaks (N on a clear peak or bridges produced by local dye saturations), and the primer region was omitted. The sense and antisense sequences obtained from the PCR-products were assembled using CodonCode Aligner (CodonCode Corporation, www.codoncode.com). For Tpm, a special treatment was performed using R (29) to distinguish

TABLE 1 | Studies selected for integrative analysis.

Reference	Co1 primer pairs for *D. gallinae* s.l.	Geographic range	Sampling date	Taxonomic range
Roy et al. (1)	COF1 + RQCOIR, CO1F4 + RQCOIR, COF1bis + RQCOIR, COF1bis + ObCOIFrev, CO1LCF + RQCOIR	France/Europe/USA	2006–2008	*Dermanyssus*
Roy et al. (28)	Same pairs without COF1 + RQCOIR and with SKPO + RQCOIR in addition	France/Europe/USA	2006–2008	*Dermanyssus*
Roy et al. (2)	Same pairs as Roy et al. (28)	France/Europe	2006–2009	*Dermanyssus*
Øines & Brännström (3)	FCOIDG + RCOIDG	Norway/Sweden/Europe	2004–2009	*D. gallinae s.s.*
Roy & Buronfosse (4)	Same pairs as Roy et al. (28)	France/Europe/Australia/Brazil	2007–2010	*D. gallinae s.l.*
Marangi et al. (20)	COX1F + COX1R	Italy/Europe	2011	*D. gallinae s.s.*
Chu et al. (22)	CO1Fyuw114 + CO1Ryuw114	Japan	2005–2012	*D. gallinae s.s.*
Pezzi et al. (25)	CO1LCF + RQCOIR	Italy	2015	*D. gallinae s.l.*
Oh et al. (21)	CO1Fyuw114 + CO1Ryuw114	Korea	2018–2019	*D. gallinae s.s.*
Ciloglu et al. (24)	COX1F + COX1R	Turkey/Europe	2016–2017	*D. gallinae s.s.*
Karp-Tatham et al. (23)	CO1Fyuw114 + CO1Ryuw114	UK/Japan/Europe	2017–2018	*D. gallinae s.s.*

COF1 (region 1, forward: 5′-ATCGGAGGATTCGGAAACTG-3′)	COX1R (region 2, reverse: 5′-TACAGCTCCTATAGATAAAAC-3′)
CO1F4 (region 1, forward: 5′-CACCTGACATGGCTTTCCCACGAT-3′)	CO1Fyuw114 (region 1, forward: 5′-AGATCTTTAATTGAAGGGGG-3′)
COF1bis (region 1, forward: 5′-CTGCACCTGACATGGCTTTCCCAC-3′)	CO1Ryuw114 (region 1, reverse: 5′-AAGATCAAAGAATCGGTGG-3′)
CO1LCF (region 1, forward: 5′-GAAAGAGGAGCAGGCACTGG-3′)	RQCOIR (region 1, reverse: 5′-CCAGTAATACCTCCAATTGTAAAT-3′)
SKPO (region 1, forward: 5′-CTTTTTAGATCTTTAATTGAAA-3′)	ObCOIFrev (region 1, reverse: 5′-GTGGGAATHGCAATAAT-3′)
FCOIDG (region 1, forward: 5′-CATTAATATTAACTGCACCTGACA-3′)	RCOIDG (region 1, reverse: 5′-CCCGTGGAGTGTTGAAATTCATGA-3′)
COX1F (region 2, forward: 5′-TGATTTTTTGGTCACCCAGAAG-3′)	COIGOR (region 1, reverse: 5′-GTTGGAATTGCAATAAT-3′)

We reviewed all available studies that have investigated the genetic structure of D. gallinae *populations using CO1 sequences. All of these studies extracted DNA from the mites individually and amplified at least one region of CO1. However, data entry into the GenBank was not performed in exactly the same way. Most authors associated each sequenced mite with a unique accession number. However, Ciloglu et al. (24) submitted haplotypes individually (only one accession number per haplotype), Marangi et al. (20) submitted part of the obtained sequences without specifying the selection criteria and Oh et al. (21) sequenced DNA from pools of mites instead of mite individuals, and they submitted one accession number per pool. The nucleotide sequences of each primer are shown at the bottom of the table.*

alleles in heterozygotes without going through a resequencing with internal primers as carried out in (4): analysis of .ab1 files with the sangerseqR (30) and Biostrings (31) packages to transform the double peak sequences into consensus sequences with IUPAC codes, then computer separation of the two alleles using Indelligent v.1.2 (32). This allowed obtaining rather coarse data which were clarified by comparing the alleles obtained in homozygotes with the alleles distinguished by Indelligent and going back and forth between alignment and chromatograms.

Processing of DNA Sequences

The sequences obtained from the databases, or from the newly generated mite collections were aligned in Seaview v5.0.4 (33) with the Clustal omega program for CO1 and Muscle program for Tpm. Each alignment was checked and corrected by hand in case of obvious local misalignment.

Given the large amount of available CO1 sequence data obtained with different sampling designs and based on primers positioned at different locations on the gene, we chose to work on haplotypes defined in two regions separately to conduct our integrative analysis. A haplotype is a unique sequence of nucleotides on a specific gene region. For mitochondrial genes, each individual carries one haplotype (two for nuclear genes) and shares it or not with other individuals. Isolating them from more or less numerous copies within populations is an essential preliminary step in population genetics to infer the evolutionary history of the gene portion concerned and compare the frequency of haplotypes between populations. By definition, the different haplotypes can only be distinguished from each other over a gene region for which data are available for all aligned sequences. When aligning together incomplete sequences (longer or shorter portions) of a given gene fragment, the non-overlapping flanking regions prevent exhaustive pairwise comparisons between all the sequences. Removing the incomplete flanking parts of the alignment makes it possible to obtain haplotypes for all the sequences. However, fewer varied nucleotide sites are likely to be encountered on a short fragment than on a longer one, resulting in a reduced proportion of different haplotypes detected in the population. Therefore, this approach is likely to provide little information on available diversity when the overlapping region is short. Alternatively, removing incomplete sequences provides a more resolved picture of the genetic diversity by allowing a more significant proportion of pairwise differences to be detected, but this reduces the benefit of large datasets. Here, to integrate all available data into the analysis while maintaining a sufficient haplotype length, in view of the location of the overlapping areas in the collected data set (**Figure 2**), we have chosen to distinguish two regions (1 and 2) and treat them independently. For the individuals with the two CO1 "regions" sequenced (isolates called DIN, JOUV,

and GZ; **Table 2**), the overlapping part was carefully examined (chromatograms + alignments) to verify *in silico* that the pairs of primers amplified the same haplotype for each individual and not different copies.

TABLE 2 | Information on new data.

Sample ID	Sampling range	Co1		Tpm	Origin
		Region 1	Region 2		
DIN	p	a	d	e	France (layer farm)
JOUV	p	a	d	e	France (layer farm)
GZ	p	a	d		France (backyard henhouse)
BE01	p	a			Belgium (layer farm)
BE02	p	a			Belgium (layer farm)
FR01	p	a			France (layer farm)
FR02	p	a			France (layer farm)
FRNP01	p	a			France (layer farm)
H	p	b′,b′′,c		e	France (layer farm)
SPT2018	p	a			France (layer farm)
SPT2020	p	a			France (layer farm)
CRZA	(p)	(a)	d		France (layer farm)
Quail	i	a			Belgium (lab quails)
Felska	i		d		Poland (layer farm)
MONT	i	b'			France (flat in a city)

*The mites were collected directly from commercial layer farms, except for GZ (private backyard poultry) and Quail (laboratory quail farm). The sampling size is considered to be at population level (p) when >15 individuals were collected from different locations in the henhouse. Otherwise, it is considered to only provide individual-level information (i). In the columns CO1 and Tpm, a letter indicates that there has been sequencing on the considered fragment and refers to the primer pair used (see **Table 3**).*

The three different datasets (two CO1 regions and one Tpm region) were isolated in Seaview in fasta format. The nucleotide sequences corresponding to the CO1 sequences obtained were translated into amino acid sequences and displayed in Seaview. Haplotypes were individualized with DnaSP v6.12.03 (34) after conversion in Seaview. The haplotype file was then converted in DnaSP to allow phylogenetic analysis in MEGA X v10.1.8 (35). Prior to phylogenetic analysis, each alignment has been the subject of a search for the best nucleotide substitution model to be used in the phylogenetic calculations using MEGA X. The phylogenetic analysis of the alignments obtained was conducted using the maximum likelihood method in MEGA X for each dataset with the model that had received the lowest AICc value (AIC with a correction for small sample sizes) in the previous search.

TABLE 3 | Primer pairs used to obtain new sequences in the present study.

Genomic region	Primer pair	Forward primer	Reverse primer
Co1 (region 1)	a	CO1LCF	RQCOIR
	b	CO1LCF	COIGOR
	b′	SKPO	COIGOR
	b′′	SKPO	RQCOIR
	c	RhipiCOIF	TyphloCOIR
Co1 (region 2)	d	COX1F	COX1R
Tpm (intron n)	e	TropoF5bisF	TropoF5bisR

*Except for primer pairs c and e, the nucleotide sequences of each primer are shown below **Table 1**. Here are the nucleotide sequences of primer pairs c and e: RhipiCOIF, 5′-CGAATAAATAATATAAGATTTTGA-3′; TyphloCOIR, 5′-GCTAATCAAGAAAAAATTTTAAT-3′; TropoF5bisF, 5′-TCGAGCACAGGAACATCACTG-3′; TropoF5bisR, 5′-AGTCTCGGCACGGTCTTCA-3′.*

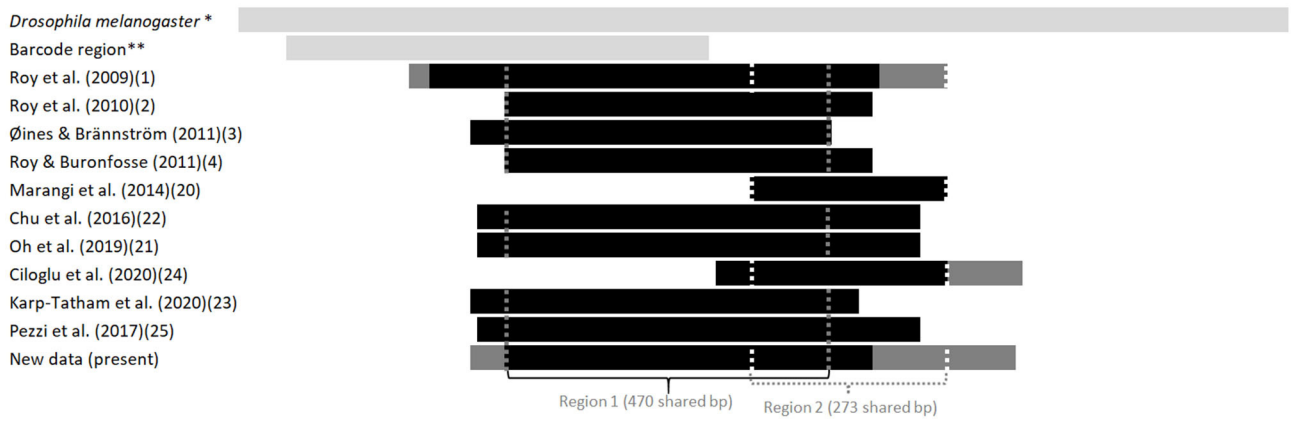

FIGURE 2 | Length and position in relation to the *Drosophila*'s full sequence of fragments of the gene coding for CO1 available in the GenBank from various studies on the genus *Dermanyssus*. The first light gray bar at the top illustrates the sequence length of the complete CDS gene in *Drosophila melanogaster*, taken as a reference, and the second one shows the length and relative position of the barcoding region. The following bars represent the positions of the sequences studied in relation to this reference sequence. Light gray, reference sequences (from studies not considered here), black, all sequences in a study; dark gray, longest sequences among sequences of various sizes (when a longer fragment has been sequenced in a part of the mites in a study). The vertical dotted lines delimit the sequences used in the two CO1 alignments analyzed in the present study (namely "region 1" and "region 2"). *NCBI Reference Sequence: NC_024511.2. ** Based on the GenBank sequence #AY956082.

In the MEGA X tree editor, labeling directly on the selected CO1 tree (one for each "region") of each haplotype from the information available in each study under scrutiny (assignment to a given group: clade, cluster and/or haplogroup) made it possible to evaluate the concordance between the different denominations and to establish the synonymies between the different denominations encountered in the literature to designate genetic groups within *D. gallinae*. On this basis, the different haplotypes identified in the present integrative analysis were assigned to clearly delineated mitochondrial groups within *D. gallinae*. The CO1 haplotype alignment nexus files of *D. gallinae s.l.* were then supplemented with geographic information (coded as traits). They were subjected to a haplotype network reconstruction using the minimum spanning network (MSN) method (36) in PopART software (37).

RESULTS

Summary of Data From the Literature

Molecular Markers

To date, investigations on the genetic structure of the genus *Dermanyssus* and/or the species *D. gallinae* have been carried out based on five molecular markers obtained by Sanger sequencing: two mitochondrial genomic regions, i.e., fragments of 16S rRNA (1, 2, 22, 27), fragments of the gene encoding CO1 (1–4, 19–26, 28) and three nuclear genomic regions, i.e., the internal transcribed spacers (ITS), including fragments of ITS1 and ITS2 and nuclear ribosomal subunits 18S, 28S rRNAs, and 5. 8S (1–3, 20–22), elongation factor 1-α (EF-1) (2) and an intron of Tropomyosin (Tpm) (2, 4).

The four most commonly used markers (mitochondrial 16S rRNA and CO1, nuclear ITS, and to a lesser extent, EF-1α) in taxonomic and phylogeographic studies provide very heterogeneous information within the genus *Dermanyssus* (1, 2), and different phylogenetic resolutions. Only CO1 was found to be truly informative in measuring intraspecific diversity in the genus *Dermanyssus* (% sequence pair differences of up to 9%) but was followed quite closely by a fragment of the gene encoding the mitochondrial ribosomal 16S rRNA. The latter shows sufficient variation to detect intraspecific structures or at the inter-/intraspecific interface (22, 27). Notably, the ITSs (ITS1 and ITS2) are very poorly informative due to the lack of intraspecific (and sometimes even interspecific) variation[1]. EF-1α, the second nuclear marker tested, was excluded from useful markers for exploring the genus *Dermanyssus* due to signs of paralogy (2).

The fifth marker used, an intron of the nuclear gene encoding Tropomyosin (Tpm), is an unconventional marker of rapid evolution, specifically developed by (2) to resolve taxonomic issues within the genus *Dermanyssus* despite the shortcomings of the two nuclear markers above. In search of a nuclear marker of rapid evolution, Roy et al. (2) amplified an intron of the gene encoding the Tpm protein whose coding regions (CDS) were available from the GenBank (accession number: AM167555.1; intron between nucleotides #495 and #496). The usefulness of this intron for exploration of genetic groups at the interface between inter- and intraspecific levels in *Dermanyssus* was found to be important as it shows a high variation of the same order of magnitude as CO1 in this species [36 CO1 haplotypes with up to 9% divergence, 39 Tpm alleles with up to 6% divergence in (2)]. In addition, more than three alleles were typically recorded within single populations (one farm or one bird nest), but only single or double sequences were encountered from single mite individuals, supporting the idea that the primers used amplify only a single orthologous locus (4).

Unlike highly variable mitochondrial genes (at least in the absence of NUMTs, as they are haploid; see above), one disadvantage associated with high variation of nuclear genes when using Sanger sequencing, is the presence of heterozygotes (individuals with two haplotypes; see the material and methods section), which results in double peak successions. To solve this problem, Roy et al. (2, 4) took advantage of the recurrent presence of heterozygous indels (indels of fixed 3-7-bp sequences) across the intron n to separate the two alleles of each heterozygous individual, as follows: (i) mapping of these indels based on sequence alignment of homozygous individuals allowed the definition of internal primers targeting the alleles with and without the different indels; (ii) the first heterozygous indel in each of the two directions was located on chromatograms from heterozygous individuals; (iii) two new sequencing reactions were conducted in each direction for each heterozygous individual to obtain the totality of the sequences of each allele. Although, somewhat tedious and expensive, this procedure has allowed refining the use of a nuclear marker to assess interspecific boundaries and to clarify the phylogenetic relationships within the genus *Dermanyssus* (19), but also to explore the genetic structure of *D. gallinae* populations (4) as well as, more recently, the genetic structure of mesostigmatic mite species belonging to the family Laelapidae (41).

Definition of Interspecific Boundaries in the Genus *Dermanyssus*

Roy et al. (1, 2) delineated the species of the genus *Dermanyssus* using a morpho-molecular total evidence approach, seeking concordant patterns between the distribution of morphological characters and both mitochondrial phylogenies and nuclear phylogenies. It is assumed here that species of the genus *Dermanyssus* all need males to develop generations (sexual reproduction at the population level), like the only species of the genus whose mode of reproduction has been studied (*D. gallinae*) as well as their close relatives *Ornithonyssus* spp. and *Ophionyssus natricis*. Indeed, the reproduction mode of all these mites is haplodiploid, i.e., males emerge from unfertilized oocytes and females from fertilized oocytes (42–44). To delineate sexually-reproducing species, the search for congruent clusters (clades) of individuals in nuclear and mitochondrial phylogenetic trees is crucial because, in most animals, the mitochondrial genome is transmitted by the mother (via the cytoplasm of

[1]This is contrary to what is known in other organisms, but consistent with what has been previously noted in other acari (38–40). This defect was confirmed in (3) and (22). These authors reported that the substantial variety of CO1 haplotypes found in hundreds of farms was accompanied by a negligible variation among ITS sequences (1 to 2 sequences max, differing by <0.4%). It is therefore unnecessary to use ITS for intraspecies investigation in *D. gallinae*.

the oocyte) whereas the nuclear genome is transmitted in a biparental manner (half by the mother and half by the father, with recombination). Consequently, within the species, because of the reticulated relationships between individuals, the evolutionary history (visualized as a phylogenetic tree) of a region of the mitochondrial genome (maternal lineage) is expected to be different from that of a region of the nuclear genome. On the other hand, between species, i.e., between entities that have not exchanged genes for a long time, the evolutionary history of the same two genomic regions is likely to be congruent. Consequently, while phylogenetic analysis of a single genomic region gives an idea of the genetic structure of a taxon, it does not allow us to identify unambiguously the points of divergence resulting from advanced speciation.

In a well-studied genus, i.e., where the interspecific boundaries have been rigorously defined on the basis of sufficient inter- and intraspecific sampling to provide a good representation of genetic variation, a gap between the largest intraspecific distance and the smallest interspecific distance is expected, referred to as the "Barcoding Gap" (45). Thus, the percentages of sequence divergence or genetic distances in pairs of individuals of the same species are generally lower than what is observed in pairs of individuals belonging to two different species. However, these values vary substantially between taxa, and there is no universal reference value for identifying interspecies boundaries based on mitochondrial topology alone, all the more when introgression occurs (46, 47). Therefore, to prevent confusion due to the presence of cryptic species and/or to NUMTs, searching in phylogenetic trees for the level of clustering of individuals that matches between mitochondrial and nuclear analysis is a reasonable way to establish interspecific boundaries [e.g., (48, 49)]. Indeed, it is expected that:

- the phylogenetic relationships between mitochondrial and nuclear sequences will be very different within the species;
- the node grouping all individuals of the species will be concordant (and not internal clades).

To observe this kind of pattern, it is necessary to work on mitochondrial and nuclear genomic regions that are sufficiently variable for the phylogenetic divergences to be informative in both phylogenetic analysis groups.

The morpho-molecular approach developed by (1) based on 46 morphological characters, two mitochondrial markers and one conventional nuclear marker (rRNA 16S, CO1, ITS) allowed a robust delimitation of the species described at that time and included in the *gallinae* group of the genus *Dermanyssus* (the other species groups were only studied morphologically) and the discovery of a species not then described: *Dermanyssus apodis* Roy, Dowling, Chauve & Buronfosse 2009. The boundaries between these species among the different populations studied are supported by the existence of segregating morphological characters (i.e., synapomorphies; stable within the species and presenting different states between species) and the concordance of groupings of individuals between mitochondrial and nuclear phylogenies. However, several strongly divergent mitochondrial lines, in the position of *D. gallinae*'s sibling group, were observed, the status of which could not be clarified due to a lack of variation in the nuclear marker ITS: clade E and clade F (= lineage L1; **Table 4**). Using the new Tpm marker (see above), interspecies boundaries among divergent mitochondrial groupings in morphologically indistinguishable *D. gallinae* mites could be explored (2). The F clade of (1), referred to as "special lineage L1," was found to be a cryptic species. *Dermanyssus gallinae s.l.* is thus a complex of cryptic species, including at least *D. gallinae s.s.* and *D. gallinae* L1 (2, 4). In contrast, the mitochondrial haplogroup named "clade E" in (1) remained classified in *D. gallinae s.s.* because it shares its Tpm alleles with the other mitochondrial groups of *D. gallinae s.s.* (2).

Finally, mites collected in farms show a particularly marked divergence between the Tpm alleles they carry (4). On farms, two to three nuclear Tpm alleles that are very distant from each other [first-level clusters in (4)] are frequently encountered with heterozygous status (often 'Tro1' and/or 'Tro2' and/or 'Tro3' alleles). In wild avifauna, heterozygotes of *D. gallinae s.s.* generally carry much less distant alleles, assigned to a single of the three groups encountered in poultry farms (2, 4). This major divergence between alleles of *D. gallinae s.s.* in farms may be the result of multiple hybridization events between incipient species (secondary fertile contacts between populations that have been separated for a long time), perhaps due to the breaking down of geographical barriers by the long-distance transfers exerted by humans. Roy and Buronfosse (4) also reported some evidence of selection effects in the allelic composition of some farm populations as can be expected from pest control treatments and other hygiene activities (reduction in the number of Tpm alleles and CO1 haplotypes).

Three atypical mitochondrial groups (highly divergent from the others) have also been reported in mites morphologically conforming to *D. gallinae s.l.* These are the JOW haplogroup, found in a poultry farm in the USA (19) and the E and F haplogroups, found in poultry farms in Turkey and Italy (24). The lack of nuclear information for these mitochondrial haplogroups made it impossible to determine the level of reproductive isolation between these mites and the others (and thus to decide whether they are separate species, intraspecific variants or pseudogenes). It should be noted that Marangi et al. (20) analyzed members of haplogroup E and members of typical haplogroups B and C from samples in Italy, but the alignment of the sequences obtained in this study (region 2) with the GenBank sequences (region 1) was of poor quality due to poor management of large non-overlapping areas by the default program used (LR pers. obs.). Consequently, while nearly half of these new sequences belonged to *D. gallinae s.s.* as shown by Ciloglu et al. (24), they all formed a group together in Marangi et al. (20), creating an artifactual double clade in the position of a sister group to *D. gallinae s.s.* + *D. gallinae* L1.

The difficulties of morphological distinction reported between species of the *gallinae*-group (18) were confirmed, with only a few discriminating characters between *D. gallinae s.l.*, *Dermanyssus carpathicus* Zeman, 1979, *Dermanyssus longipes* (Berlese and Trouessart, 1889), *Dermanyssus hirundinis* (Hermann, 1804), *D. apodis,* and many unstable characters within species (1). In particular, the distinction between *D. gallinae s.l.* and *D. apodis*

TABLE 4 | Correspondence of the different identifiers used in the studies included in this analysis to designate mitochondrial groups and their taxonomic positioning determined by comparing mitochondrial and nuclear data.

Identifiers in the different studies								Taxonomic positioning
RO0	**RO1**	**OB**	**RB**	**CHU**	**KT**	**CIL**	**Present**	
Clade E	Co21 & Co22						Co21-22	NUMTs
	JOW						JOW	NUMTs
	Lmt3	A	Lmt3	A	A	A	A	*D. gallinae s.s.*
	Lmt2	B	Lmt2	B	B	B	B	*D. gallinae s.s.*
	Lmt1	C	Lmt1	C	C	C	C	*D. gallinae s.s.*
clade F	L1	D	L1	D	D	D	D	*D. gallinae* L1
						E	E	NUMTs
						F	F	NUMTs
							H	NUMTs

The identifiers placed on the same line refer to the same genetic groups. The study by (20) is not included because the phylogenetic reconstruction presented does not allow a correct assignment of sequences to the identified groups (see text). This lack of assignment was corrected in (24), who integrated part of the data from (20). The right-hand column ("present") proposes to set the simplest identifiers for use. RO0, (2); RO1, (2, 19, 28); OB, (3); RB, (4); CHU, (22); KT, (23); CIL, (24). NUMTs, "nuclear mitochondrial DNA" (artifactual genetic group, not to be taken into account).

is based on discriminant traits that are challenging for non-specialists to visualize (using a light microscope). Of course, the unavailability of morphologically discriminating characters within *D. gallinae s.l.* makes the morphological distinction between *D. gallinae s.s.* and *D. gallinae* L1 impossible (hence the term, cryptic species). However, since only *D. gallinae s.s.* has been found to be present in poultry farms in Europe and several parts of the world, the hematophagous mites to be distinguished in poultry farms in Europe and possibly in the world can be reduced to two very distant species *D. gallinae* (Dermanyssoidea: Dermanyssidae) and *Ornithonyssus sylviarum* (Canestrini & Fanzago, 1878) (Dermanyssoidea: Macronyssidae) (19). Therefore, the well-illustrated key proposed by Di Palma et al. (50) to distinguish *D. gallinae* from *O. sylviarum* is fully operational for studies restricted to layer farms, where *D. gallinae* is a problem. On the contrary, it may not be suitable for investigations in wild birds or even in other types of farming (pet bird farms, game farms, etc.).

Integrative Analysis of Sequence Data

The sequencing of portions of the CO1 gene with different pairs of primers in the same mite individual in the present study (**Tables 1**, **2**) did not reveal the presence of different copies, except for one individual (farm H). Identical copies were regularly amplified with different pairs since identical haplotypes are recurrently found in other studies. These elements make it possible to legitimize the comparison between sequences obtained in the different studies.

Three mitochondrial lineages known since 2011 are designated by two series of identifiers (mitochondrial lineage Lmt*n* or haplogroup *N*) that revealed to be exactly synonymous (**Figures 3**, **4**; **Table 4**). The mitochondrial lineages Lmt1, Lmt2, and Lmt3 determined by phylogenetic reconstruction by Roy et al. (2) and defined as second-level clusters by Bayesian clustering by Roy & Buronfosse (4) match exactly the haplogroups C, B, and A defined by Øines & Brännström (3) by phylogenetic reconstruction and taken up by Chu et al. (22) and Ciloglu et al. (24) (**Supplementary Material 1**). These three lineages, widely represented in the different studies, will be referred to in the rest of the text as "typical haplogroups" of *D. gallinae s.s.* and the letters A, B, and C will be used to name them.

Four atypical haplogroups in mites morphologically conforming to *D. gallinae s.l.* have been reported in the literature, namely JOW in (19), 'Co21-22' in (2), haplogroups E and F in (24) (see above), and a fifth appears in the new data, namely haplogroup H. Of these, four have been encountered in poultry farms: JOW (in the USA), E and F (in Italy and Turkey), H (in France). In contrast to the three typical poultry and cosmopolitan haplogroups detailed above, at least the last three (E, F, and H) show a much narrower distribution, as they have only been found occasionally in regions of Europe that have been explored in detail (Turkey, Italy) and where typical haplotypes are encountered recurrently [see (23) and **Figure 3**]. Predictive translation of the nucleotide sequence revealed that the sequences of haplogroup H contain an indel followed by three stop codons. The other CO1 sequences (region 1 and region 2) do not contain any other stop codon; however, substantial differences in the aminoacid sequences translated from the obtained DNA sequences exist between the atypical haplogroups E and F on the one hand and the whole *Dermanyssus* sequences, including *D. gallinae s.l.,* and the outgroups on the other hand (**Supplementary Material 2**). Considering all positions of the nucleotide sequences, these atypical haplogroups, although, carried by mites morphologically conforming to *D. gallinae s.s.*, were located outside this species, amongst the outgroup species of the genus *Dermanyssus*, according to the phylogenetic ML inference: with the region 1 dataset, the JOW haplogroup was located in the middle of the clades of outgroups (**Figures 3**, **4**) and with the region 2 dataset the atypical haplogroups E and F form a sister clade to *D. gallinae s.l.* + *D. apodis*. Considering only the first position of the codons, the resolution of typical haplogroups decreased drastically in accordance with what is

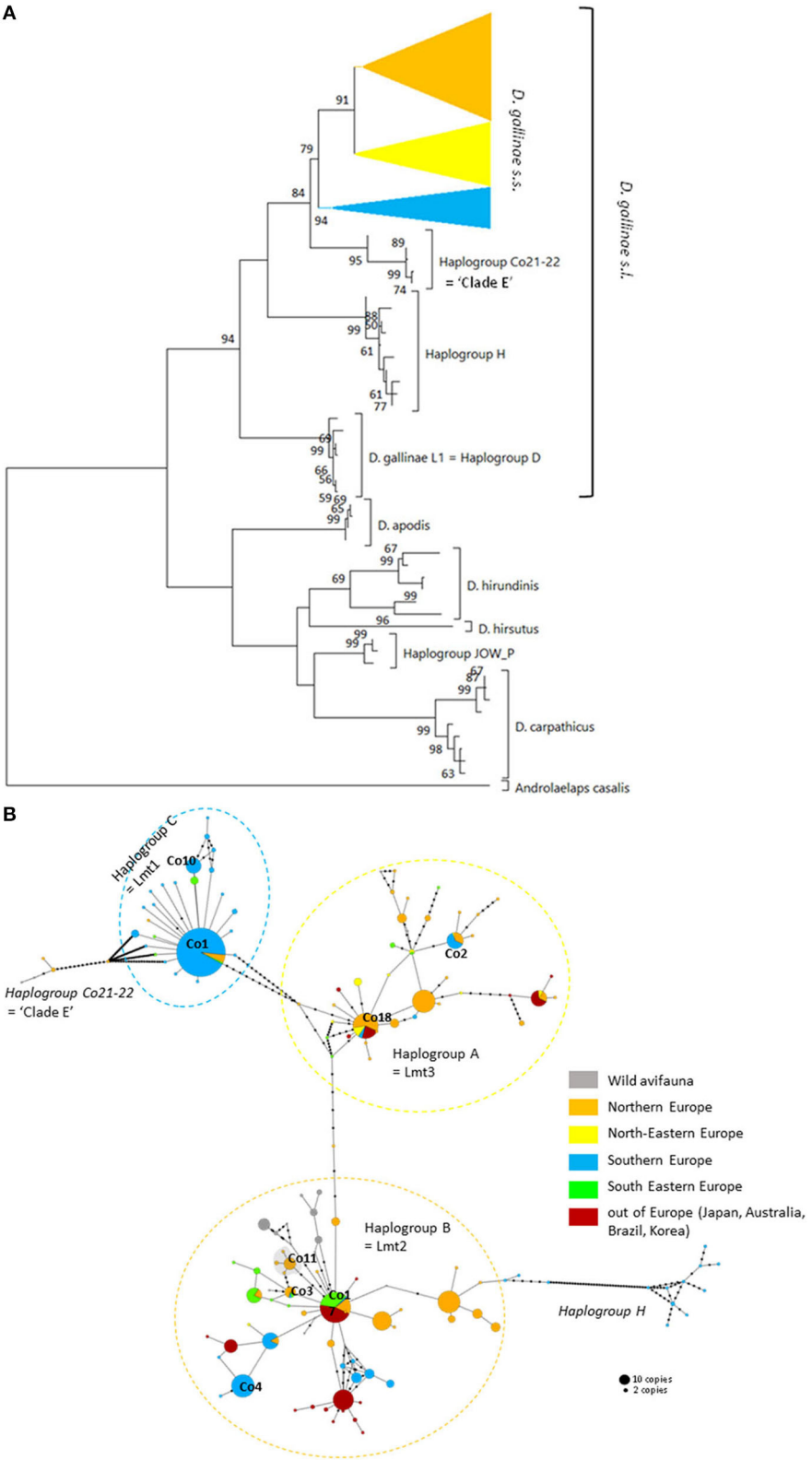

FIGURE 3 | Genetic structure from CO1 haplotypes on region 1. **(A)** Evolutionary history of the different region-1 haplotypes obtained to date from the genus *Dermanyssus* with condensed clades within *D. gallinae s.s* (132 sequences) and *Androlaelaps casalis* (Mesostigmata: Dermanyssoidea) as an outgroup. A single copy

(Continued)

FIGURE 3 | of each haplotype has been integrated to the analysis (176 nucleotide sequences; alignment 470 bp long). It was inferred by using the Maximum Likelihood method and General Time Reversible model (51). The tree with the highest log likelihood (−4378.86) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories; +G, parameter = 0.4639). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 25.74% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values >50% are displayed at the nodes. **(B)** Haplotype network (MSN) of the haplotypes in *D. gallinae s.s.* (*D. gallinae s.l.* excluding *D. gallinae* L1). Dashed lines delineate the three main haplogroups of *D. gallinae s.s.* recorded from region-1 data (the colors correspond to those of the condensed clades in **(A)**). The two atypical haplogroups in this dataset are indicated in italics. The size of the disks is proportional to the number of sequences present in the database and the IDs of some of the haplotypes isolated in (2) appear next to or on the disk. The four haplotypes in the shaded area including 'Co11' are proper to the lab SK population. Geographical locations in Europe are as follows: Northern Europe (orange) includes Belgium, UK, Denmark, Norway, Sweden, Finland, The Netherlands; North-Eastern Europe (yellow) includes Poland and Czech Republic; Southern Europe (blue) includes France, Italy, Portugal; South Eastern Europe (green) includes Albania, Croatia, Slovenia, Turkey, Greece. Haplogroup C seems to be mainly present in Southern Europe, whilst Northern Europe and the present non-European countries are dominated with the haplogroups A and B. Black dots along the links between disks = mutations.

expected for a functional genomic region, and the resolution of the three atypical haplogroups remained high, with long and strongly supported branches (**Figure 5**). Lastly, Tpm sequences obtained from mites carrying the haplogroup H share Tpm alleles of mites carrying typical haplogroups of *D. gallinae s.s.* (**Supplementary Material 3**).

DISCUSSION

Typical Mitochondrial Haplogroups A, B, C and Signs of Expansion Within *D. gallinae* s.s.

The three typical haplogroups show recurrent signs of expansion: several star patterns in haplotype networks are formed by multiple rare variants very closely related (1–2 mutations) to a strongly represented central haplotype [in particular haplotypes respectively named 'Co1,' 'Co17,' 'Co18' in (2, 4) and **Figure 3B**]. This is consistent with large populations that develop rapidly from a small number of individuals after undergoing a drastic reduction (bottleneck events) or after a small number of individuals have colonized a new area (founder events), thus growing from only a few haplotypes if not a single one. The former may typically happen after a massive acaricide treatment and the latter after contamination of an uninfested farm. Over many generations, if kept isolated, such a population will only contain the initial haplotype(s) and a few closely related recent variants, i.e., haplotypes emerging by random point mutation within the population. The low local variety of haplotypes supports this hypothesis, with almost always a single haplotype being the dominant haplotype within a poultry house or group of nests [(3, 4, 22), new data, **Figure 6**]. This type of process well explains the three-point star-like structure formed in the CO1 haplotype network by haplotypes isolated from the laboratory population SK ('Co11', **Figure 3**). It was observed that after mites were collected from a Danish layer farm, this SK-population underwent a marked bottleneck event during the initiation of its culture in the laboratory (Kilpinen, pers. comm.). This population was then grown in the laboratory for more than a decade (between 1997 and 2009), maintained in inbreeding (without adding new mites) over >430 generations (assuming three generations per month). During this period, it seems like three new haplotypes appeared, differing by one nucleotide each from the majority one, as a result of three independent mutation events. The probability that such an inheritable mutation (a random event during gamete formation) will appear in the gene region under consideration is naturally increased by the population's size. The much larger population size on the scale of interconnected farms compared to a laboratory facility explains well the much higher number of closely related variants (stars' points) of the haplotypes 'Co1,' 'Co17,' 'Co18.' Oh et al. (21) detected exclusively the 'Co18' haplotype in 13 Korean farm buildings, which might suggest a very recent invasion in these farms. However, as these authors carried out only one sequencing per building, the rare recent variants may have been missed.

Although, the typical composition of a farm population is centered around a single major haplotype with a small number of close variants, in some cases, two or even three typical haplogroups have been encountered at the same time in single laying hen facilities (4, 23, Øines pers. comm., present study). From a diagnostic point of view, this substantially increases the number of segregating sites per haplotype S/h within the poultry house, without necessarily increasing the haplotypic Hd diversity (**Figure 6**). This pattern can be explained by a new contamination event in an already infested farm, producing secondary contact between long separated populations (see below).

Atypical Haplogroups E, F, H = NUMTs Pseudogenes

From *in-silico* analysis of the sequences in the JOW, CO21-22, E, F, and H haplogroups, we can conclude that these are clearly non-functional given the important differences on positions 1 and 2 of the codons, including the presence of one stop codon, in the haplogroup H. While the existence of stop codons in a protein-coding sequence is a strong indication of loss of functionality, their absence (as in JOW, CO21-22, E, and F) is not evidence of functionality (54). On the other hand, the persistence of the strongly supported clades of atypical haplogroups, while those of typical haplogroups disappear when the third positions are omitted (comb-like; **Figure 6**), supports the loss of functionality in the former. Since most of the synonymous nucleotide substitutions (which do not produce an amino acid change, therefore more likely to be a substitution which does not affect the fitness of the organism) are on position 3, it is consistent to no longer perceive differentiation when

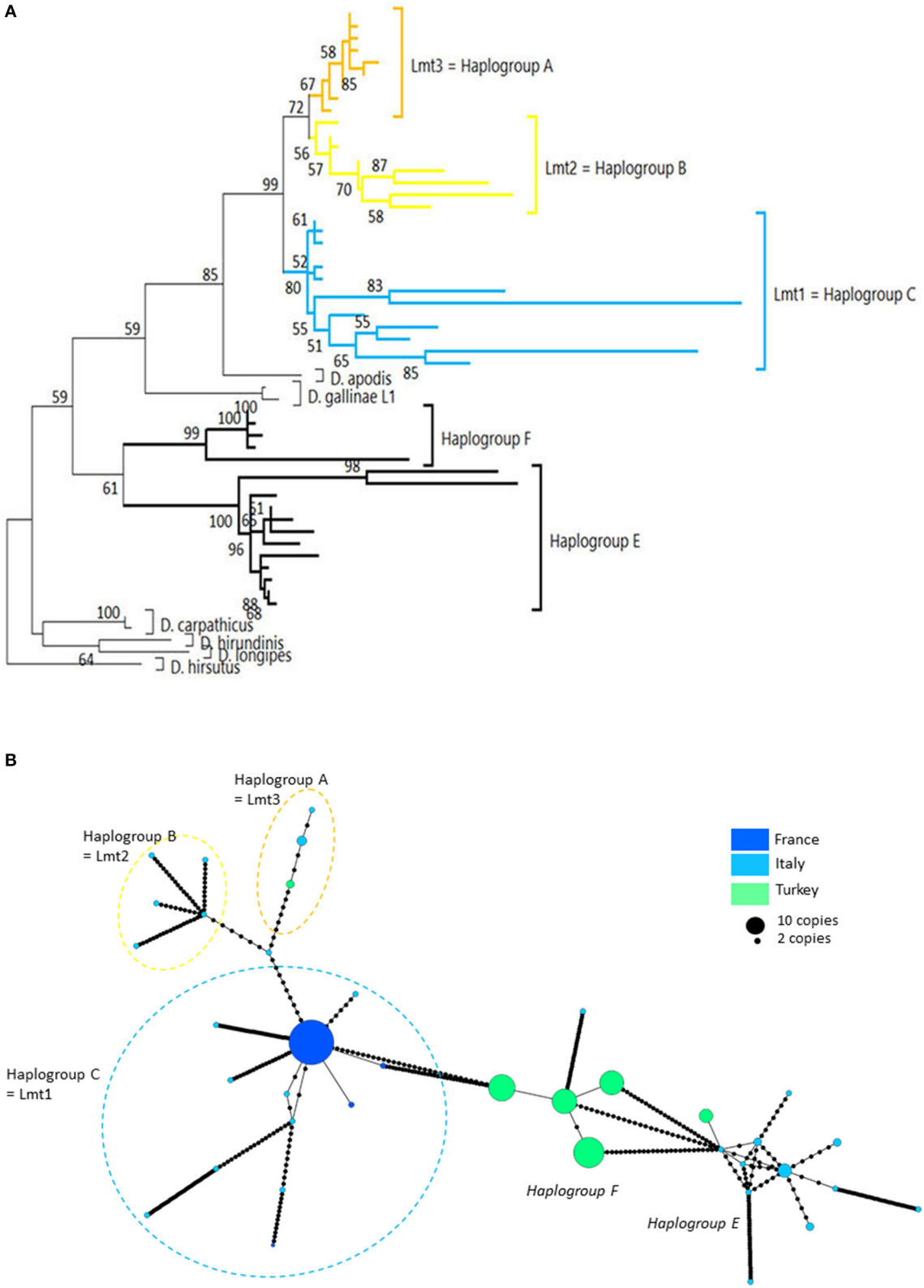

FIGURE 4 | Genetic structure from CO1 haplotypes on region 2. **(A)** Evolutionary history of *Dermanyssus gallinae s.l.* CO1 haplotypes based on all available data on the region 2 of CO1 (273 bp) using the Tamura 3-parameter model (52), with sequences of other *Dermanyssus* species as outgroups (*D. apodis*, *D. carpathicus*,

(Continued)

FIGURE 4 | *D. hirundinis*, *D. longipes*, *D. hirsutus*). A single copy of each haplotype (55 nucleotide sequences) has been integrated to the analysis. The tree with the highest log likelihood (−2975.33) is shown. Only bootstrap values >50% are displayed at the nodes. In order to be able to root the tree with outgroups and to obtain a sufficiently informative topology, we have integrated sequences that are not completely overlapping into the analysis (2, 3, 20, 24), new data). The non-overlapping areas have been supplemented by *N* in the alignment. **(B)** Haplotype network (MSN) from CO1 region-2 data. In order to work on haplotypes of reasonable size that are completely resolved in this genomic region with some representation of the population structure, we have integrated into the analysis exclusively the data from (20, 24) (Turkey) and the present data obtained for region 2 (four populations; France). Dashed lines delineate the only typical haplogroups of *D. gallinae s.s.* recorded from region 2 data (the colors correspond to those of the condensed clades in **(A)**). The two atypical haplogroups in this dataset are indicated in italics. The size of the disks is proportional to the number of sequences present in the database and the name of one haplotypes identified in (2) appears on the disk ('Co1'). Black dots = mutations.

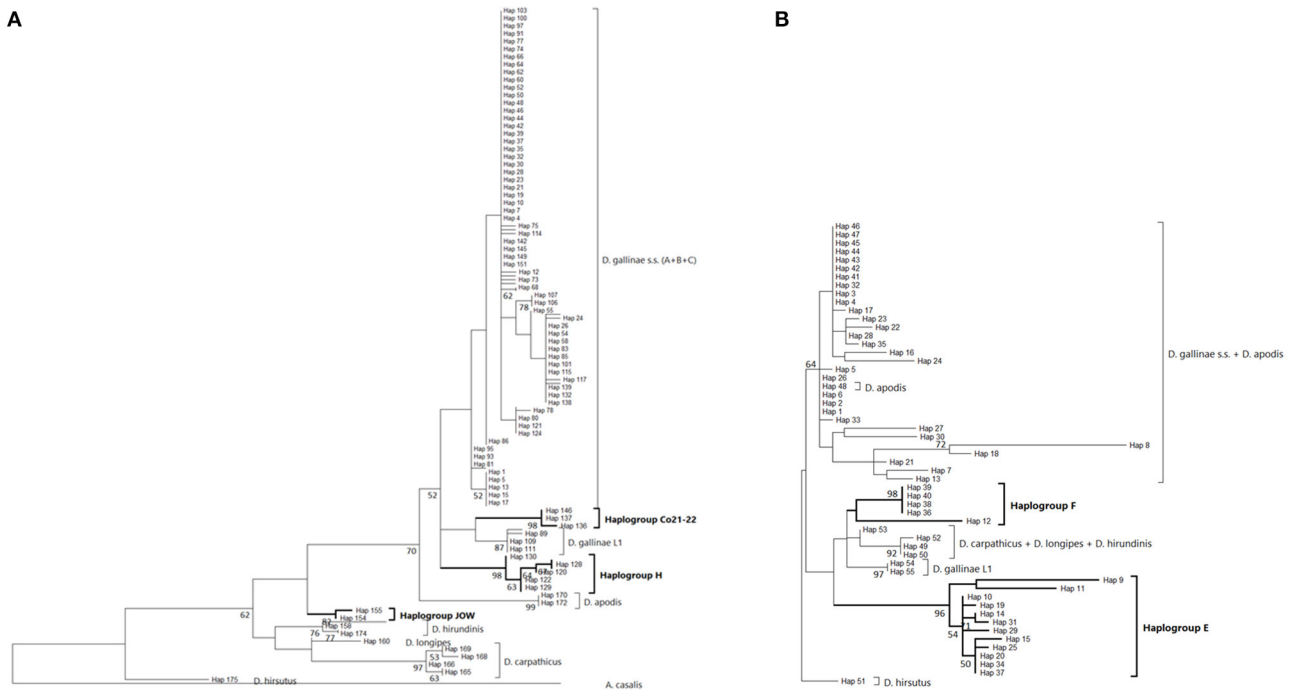

FIGURE 5 | Evolutionary history of *Dermanyssus gallinae s.l.* CO1 haplotypes based on 1st codon positions of available CO1 data using the Maximum Likelihood method. A single copy of each haplotype has been integrated to the analyses. Only bootstrap values >50% are displayed at the nodes. In bold, atypical haplogroups. **(A)** Region-1 dataset (176 nucleotide sequences and 157 positions). The General Time Reversible model (51) was used with a discrete Gamma distribution to model evolutionary rate differences among sites (five categories (+*G*, parameter = 0.4599) and the rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 31.21% sites). The tree with the highest log likelihood (-813.96) is shown. **(B)** Region-2 dataset (55 nucleotide sequences and 91 positions). The Tamura-Nei model (53) was used with the rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 17.58% sites). The tree with the highest log likelihood (−737.98) is shown. Note: the longest branches in the *D. gallinae s.s.* + *D. apodis* clade are due to sparse differences in the haplotypes belonging to the typical haplogroups from Marangi et al. (20), likely due to double sequences (haplogroup E + typical haplotypes).

neglecting position 3 of functional sequences of closely related organisms. Conversely, the maintenance of groups formed by other sequences is explained by the relaxing of the selection pressure following the loss of their functionality. At higher taxonomic levels, non-silent substitutions can, however, provide valuable information on phylogenetic relationships between distantly-related organisms: removing the third position can help address compositional heterogeneity and saturation, which may occur due to frequent changes in the third codon position in datasets of CO1 from organisms whose divergence is ancient and can lead to long-branch attraction (55). The 4 NUMT lines found in the present study seem to originate from different ancestral haplotypes, which suggests that several events of incorporation into the nuclear genome of *D. gallinae* may have occurred independently, as has been reported in some insects (56). Lastly, the pseudogenic status is corroborated by the absence of evidence of advanced speciation in the Tpm nuclear DNA (alleles are shared between atypical-H mites and typical-A-B-C mites). For comparison, the reproductive isolation of *D. gallinae* L1 is supported both by a long and strongly supported mitochondrial branch (**Figures 3**, **4**) and by original nuclear Tpm alleles, i.e., not shared with any of the mites carrying typical *D. gallinae s.s.* haplotypes (**Supplementary Materials 3**, **4**). All these sequence characteristics of stop-free atypical haplogroups strongly suggest that they are also NUMTs, even if the relaxation of the selection pressure has not yet resulted in the occurrence in the present sample of stop codons in all of them.

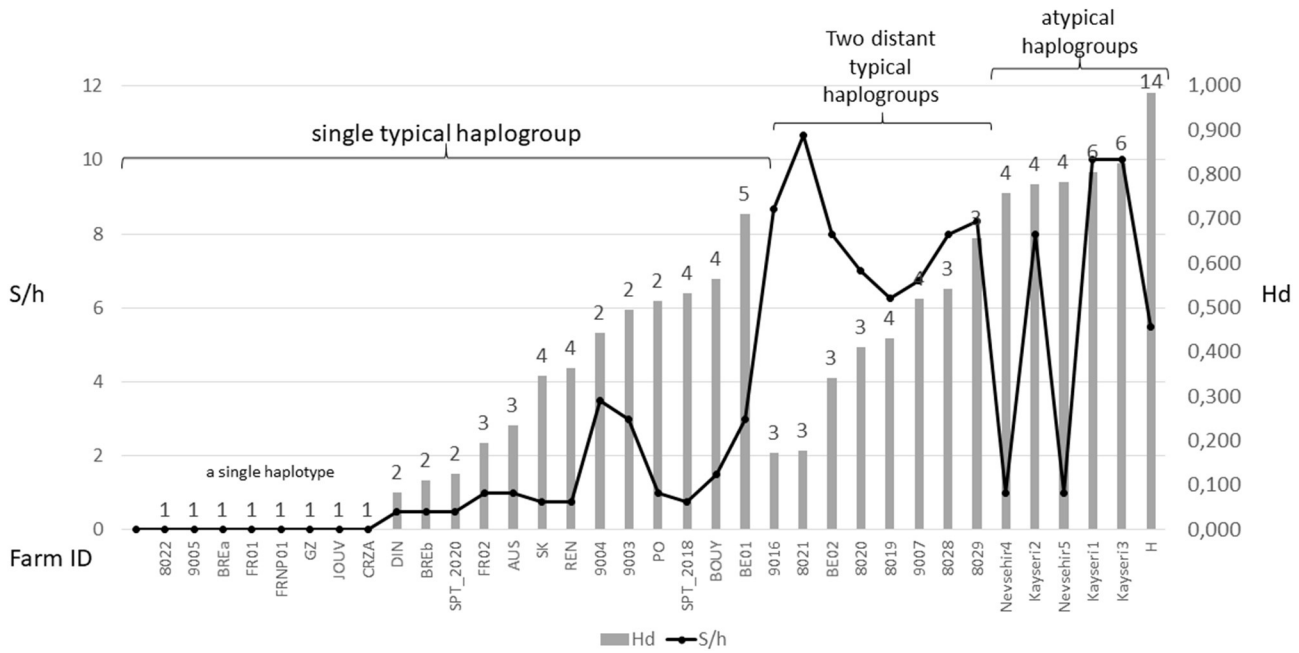

FIGURE 6 | Overview of diversity indices based on CO1 haplotypes in *D. gallinae s.s.* populations encountered in farms. The numbers above the bars represent the number of haplotypes encountered in each farm sample. Hd, haplotype diversity; S/h, ratio of the number of segregating sites to the number of haplotypes detected in an isolate. Values are ordered from left to right within three different mt profiles: populations carrying a single typical haplogroup, populations carrying two or three typical haplogroups including C and populations carrying any atypical haplogroups. The data set is presented in **Supplementary Material 3**. High S/h values were always recorded in farms where haplogroup C and one or two of the other haplogroups were found together, regardless of the Hd value. High Hd values (>0.75) were found in farms where atypical haplotypes have been amplified (haplogroups E, F, and H), likely due to the rapid evolution of the non-functional sequences.

At the population level, the frequency and spatial distribution patterns of atypical haplogroups further consolidate this hypothesis. The atypical haplotypes encountered in the farms sampled at population level by (24) (E, F) and in the present study (H) show very different demographic profiles from typical haplotypes: several related variants are present within the same henhouse with relatively balanced frequencies (absence of star pattern, **Figures 3**, **4**; higher Hd value than in farms without atypical haplogroup, **Figure 6**). The rapid evolution of non-functional sequences probably explains this scattered pattern different from the expansion patterns found in typical haplogroups. For the atypical haplogroups JOW and Co21-22 (clade E of (1) + new "Quail" isolate), the limited sampling does not provide an accurate representation of the population and thus demographic profiles. However, the fact that 3 different haplotypes were found among 4 individuals in the new "Quail" isolate and the JOW isolate suggests a similar distribution pattern to the other atypical haplotypes.

Interestingly, all the sequences provided by (20), which were not linked to haplotype E and were assigned here to the typical haplogroups A, B, and C show a clear excess of non-synonymous mutations when considering the corresponding amino acid sequences (**Supplementary Material 2**). This excess translates into excessively long branches and links in the phylogenetic tree (**Figure 4A**) and the network of haplotypes (Italy in **Figure 4B**), respectively. This could be explained by double peaks in the chromatograms, generated by the simultaneous amplification of a NUMT with its mitochondrial counterpart, as was observed in the present study with mites from the H isolate (six individuals out of 20 discarded from the analysis because of too poor quality chromatograms due to the presence of a multitude of double peaks). Here, the absence of indels makes it impossible to deduce the two sequences by Indelligent analysis (search for the repetition of sequence portions with a shift of the length of the indel) or internal sequencing as was done for Tpm (4). This is why we warn prospective researchers against using such chromatograms for future mite studies.

Ecological and Geographical Distribution of the Genetic Groups of *D. gallinae s.l.*

Dermanyssus gallinae s.s. has a much broader host range than other species within *Dermanyssus*. It is the only species encountered in birds belonging to different taxonomic orders [at least nine orders according to (28)]. It is also the only species encountered in poultry farms to date. All species of *Dermanyssus* other than *D. gallinae s.s.* have been recorded in a single family or genus of bird or a small number of species of the same order nesting in the same environments and conditions (28). The cryptic *D. gallinae* L1, although morphologically indistinguishable from *D. gallinae s.s.*, also has a narrow host range: it was found almost exclusively in pigeons, both in wild (urban) nests and pigeon farms, in both Europe and the USA. Many morphology-based studies have shown that mites

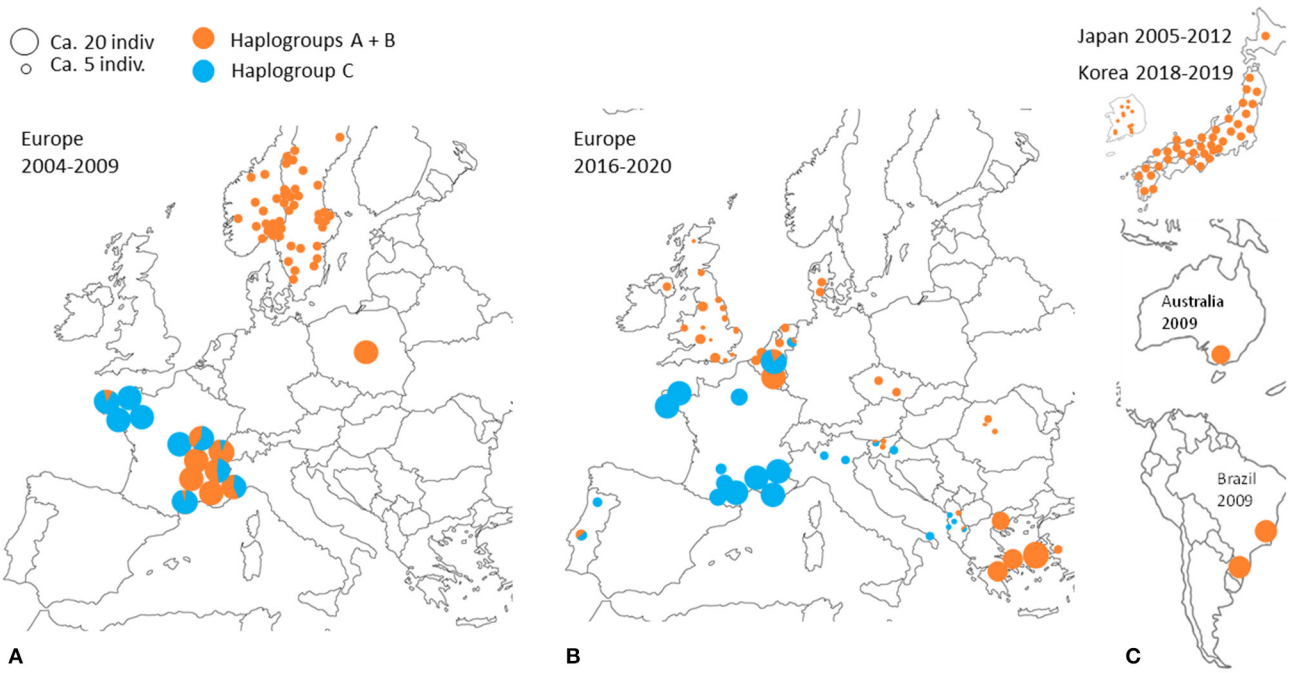

FIGURE 7 | Geographical distribution of typical CO1 haplotypes A+B and C at different periods according to data from (3, 4, 21–23). A disc represents a set of mite individuals collected from the same poultry house (or sometimes from several poultry houses within the same geographical entity) and the colors indicate the proportion of individuals carrying haplotypes A or B (orange) and individuals carrying haplotypes C (blue). The size of the disc is proportional to the number of individuals sequenced. **(A)** Previous decade in Europe (2004 and 2009). **(B)** Current decade in Europe (2017–2020). **(C)** Previous decade and the beginning of the current decade in other regions of the world.

belonging to *D. gallinae s.l.* also occasionally bites humans [see (57) for review]. Amongst them, in the few morpho-molecular studies published so far on human cases, *D. gallinae* L1 has been shown to cause human disease in urban areas on at least two occasions (25, 27). Conversely, a haplotype of *D. gallinae s.s.* typical of layer farms has been reported in humans in a third case report (26). The One Health potential of this mite should not be ignored, not only as being the cause of human gamasoidosis, but perhaps more importantly, with an unknown impact by its vector capacity, for poultry and humans as several bacterial and viral pathogens has been described from it.

In poultry farms, the three typical haplogroups of *D. gallinae s.s.* are widely represented in Europe, as well as in the other regions of the world studied so far [Japan (22), Korea (21), Brazil, and Australia (4)]. This wide distribution is almost exclusively related to human activities: populations that develop in wild avifauna have been shown to be highly isolated reproductively (3, 4). However, a geographical structure is emerging since haplogroup C is present mainly in southern European countries and haplogroup B in northern European countries (**Figure 3B**). Haplogroup A is the most cosmopolitan. Within the three typical haplogroups of *D. gallinae s.s.*, Øines & Brännström (3) detected signs of differentiation between populations collected from Swedish and Norwegian farms consistent with the administrative functioning of these two Scandinavian countries: series of closely related haplotypes are present in both countries, but none are shared.

On the other hand, Swedish farms share haplotypes with the rest of Europe. This is consistent with foreign trade being much more developed in Sweden as it is part of the European Union, where there is an open policy of movement of goods and services, while Norway not being part of the EU. The tariff protection on meat and egg products between Norway and its European neighbors ensures a good domestic market for Norwegian domestic production of agricultural products, implemented by the Norwegian government (58). This legislation helps control the import of live chickens to reduce the import of poultry diseases (59). With a generally increased burden of bureaucracy and documentation need when importing animals, this is likely to limit the flow of potentially contaminated products or animals. This trade barrier may have contributed to Norway having one of the lowest rates of mite-infected layers in Europe (60).

Despite the motley and irregular nature of the available sampling, the accumulation of information on the European distribution of haplogroups of *D. gallinae s.s.* by successive studies and our current data shows variations in time and space consistent with a massive expansion process in Europe of haplogroup C from a point of origin in or near France (**Figure 7**). A major socio-economic reorganization of the poultry industry around Lyon in France late 1990's might have generated the mixing between two strongly differentiated genetic groups (haplogroup C vs. haplogroups A+B, A and B being more closely related to each other): the distance traveled by trucks transporting spent hens was abruptly increased as a result of a

shift from a configuration with multiple local slaughterhouses to an arrangement with large centralized slaughterhouses in regions far from Lyon, particularly in Brittany (and outside France; Lubac, ITAVI, pers. comm.). Ca. 10 years later, Roy & Buronfosse (4) observed in 2008–2009 several populations with a mixed mitochondrial profile in these two distant French regions. The inverted proportions of haplogroup C vs. A+B (haplogroup C majority in Brittany (NW France) and haplogroups A and/or B globally majority around Lyon (SE France); **Figure 7**) suggested a recent transfer of haplogroup C from Brittany to Lyon's region. During the last decade, haplogroup C seems to have expanded from Brittany to the SE zone of France via poultry transport within France. During the current decade, haplogroup C remains concentrated in France, but is also found in surrounding countries (Portugal, Italy, Belgium, the Netherlands).

Interestingly, the other typical haplogroups have not been found in France during the last decade, whereas they were frequent in the previous decade sampling. The disappearance of the other haplogroups in samples from France in the current decade suggests an intraspecies competitive exclusion effect or particularities favoring the persistence of haplogroup C in hybrid populations [cytoplasmic incompatibility due to a bacterium, for example; (61)]. The concomitant presence of this haplogroup and another typical haplogroup in a Belgian henhouse, as well as in four other sampling points in Portugal, the Netherlands, Slovenia and Albania, suggests that a process of geographic expansion is underway in the countries around the probable country of origin: new contamination events seem to bring haplogroup C into contact with other haplogroups in France's neighboring countries. This apparent expansion is consistent with the demographic profile of expansion observed around the 'Co1' haplotype in the CO1 network of **Figures 3**, **4** and is consistent with its relatively recent local origin in France.

CONCLUSIONS, PERSPECTIVES, AND RECOMMENDATIONS FOR FUTURE STUDIES

This study provides a framework for future studies requiring knowledge about interspecific boundaries (who is who?) and spread routes (who goes where?) of PRM. Layer farms in the parts of the world studied so far are infested by three mitochondrial haplogroups (A, B, C), one of which appears to be expanding (C). It would be interesting to explicitly test the hypothesis of an ongoing invasion by this haplogroup and to study the effects of hybridization between haplogroups A, B, and C. Indeed, if, as suggested by the spatio-temporal distribution of the haplogroups in the available data, the genetic structure of the populations infesting the farms in the northern parts of Europe is indeed being modified, changes in the response to treatments or the effect on the animals could take place in the coming years depending on the respective characteristics of these genetic groups and the effects of their possible hybridization. To anticipate any possible changes in the behavior of *D. gallinae s.s.* infestations, it would therefore also be advisable to verify that these haplogroups are indeed interfecund and to study the differences in treatment resistance, pathogenicity and/or vector capacity between haplogroups and/or between hybridized and not hybridized populations.

Our study also points out that it is possible that NUMTs can confound the analysis and methodological aspects when studying variations in mitochondrial sequences in *D. gallinae* and calls for the researcher to be vigilant and perform necessary QC actions on the data. The absence of stop codons may not be sufficient to ensure that the amplified sequences are, in fact the targeted mitochondrial sequences when working with the gene encoding CO1. It is recommended to sequence individuals separately, perform this on several individuals per henhouse, and to consider several criteria to avoid pollution of the analysis by NUMTs, especially when working on region 2 as our analysis indicate it is the portion of CO1 in which the most significant proportion of individuals have resulted in the sequencing of NUMTs so far. In case of divergence from the CO1 sequences of the three mitochondrial haplogroups typical of *D. gallinae s.s.* (or *D. gallinae* L1) in mites whose morphology conforms to *D. gallinae* according to (1), or simply according to (50) in the case of sampling from layer farms, the following indicators constitute warnings:

1. Amino acid CO1 sequence showing several segregating differences (within the sample) with respect to the sequences of *D. gallinae* s. l. individuals available in the GenBank and/or the maintained resolution of the new grouping in phylogenetic analyses conducted on the first codon positions only (whereas, the groupings of A, B, and C are lost).
2. Discrepancies between CO1-based phylogenetic trees and the taxonomic framework. Integrating the sequences of several species of the genus *Dermanyssus* (if possible even several individuals per species; to be collected from the GenBank) into the phylogenetic analysis allows to visualize the positioning of the tested sequences in relation to that of the other species and to detect possible discrepancies such as those found with the haplogroup JOW.
3. Multiple related CO1 haplotypes within a building with a more or less balanced frequency of each (Hd value >0.70). This feature requires a quite intensive sampling effort (ca. 20 individuals per henhouse) to be assessable.
4. Careful examination of chromatograms of CO1 sequences containing series of polymorphic sites and double peaks. Care when using cloning methods in conjunction with sequencing as a wrong copy may be characterized. This may result from concomitant amplification of both the functional mitochondrial and NUMT sequences if performing PCR on DNA from a single mite.
5. Nuclear alleles of variable regions (e.g., Tpm, microsatellites...) shared with mites carrying typical haplotypes.
6. We warmly encourage those planning to conduct further studies on the genetic structure of *D. gallinae* to check at least warnings #1 to #4, and ideally, in case of doubt, to supplement the analysis with nuclear markers (warning #5). In addition, it is recommended to work preferentially on carefully preserved mites because it has been found in other arthropods that NUMTs were more often amplified from long-dried

individuals (14). Zhang et al. (62) noticed that in some dried insects, the nuclear sequences were preferentially amplified instead of their mitochondrial counterpart. Therefore, these authors and Leite (14) recommend to work if possible on fresh specimens or at least on well-preserved ones. Ideally, mites are placed alive at −20°C after a few days at RT° to allow blood digestion (since blood contains esterases that can disrupt PCR amplification) either dry or in ethanol at >95°C.

In addition, given that gamasidosis is becoming a growing One-Health problem for both humans and other mammals (57, 60), that mites associated with pigeons or hens have been identified as the culprits, and that the distinction between *D. gallinae s.s.* and *D. gallinae* L1 can only be made on a molecular basis, the definition of a PCR diagnostic protocol in human and veterinary medicine could prove useful to enable practicing veterinarians, physicians, dermatologists, acarologists, to identify the source of human gamasidosis clearly.

Finally, based on the present state of the knowledge acquired using the Sanger sequencing method, several promising research directions can be taken by capitalizing on two major advances:

- Complete genomes of a few *D. gallinae* isolates, one nuclear and one mitochondrial, have recently been released in public databases: GenBank assembly accessions GCA_003439945.1 [nuclear genome, (63)], MW044618.1 (complete mitochondrial genome).
- High-throughput sequencing technologies are improving substantially while becoming increasingly affordable.

The availability of the complete nuclear genome of PRM opens major prospects for the development of microsatellite and/or SNP markers, rapidly evolving markers more appropriate than nuclear Sanger sequences for intraspecific analysis thanks to analytical methods allowing easy management of heterozygotes. In addition, while barcoding allows rapid assignment of individuals to species in relatively well-studied taxonomic groups based on a single genomic region, i.e., a single locus, relying on one or two loci to determine the neutral genetic structure of populations within the species and to analyze spread routes has significant drawbacks. Even if the said markers (here CO1 and Tpm intron) extend over several hundred nucleotides, have high intraspecific variability and are likely to be subject to little selection effect, these are in the end single loci, the different nucleotidic sites being strongly linked to each another. There is, therefore a significant risk that their variation does not provide sufficiently reliable evidence of the evolutionary history of the genomes of which they constitute a very small sample. The heterogeneity of the differentiation of the different loci within the genome between populations is now known, resulting from the contrasting effects of natural selection (divergent differentiation), gene flow (homogenization) and variations in mutation rates across the genome [see (64)]. Thanks to genomic data, one can now easily design a few dozen microsatellite markers or SNPs randomly distributed in the genome of *D. gallinae* and compare multilocus genotypes from different populations. This is one more reason to consider that future microsatellite markers and/or SNPs will bring a lot.

High-throughput sequencing technologies that are becoming increasingly affordable (e.g., Illumina sequencing to obtain many short sequences or nanopore sequencing to obtain very fast long sequences) will allow even more rapid development of multilocus markers, using, for instance, restriction site-associated DNA sequencing [RAD-seq; (65)]. These advances should allow us to refine our understanding of the expansion process of haplogroup C and consequently develop relevant recommendations to improve prophylaxis in farms in different European countries. However, when using NGS approaches, there should be care taken on the dataset, especially when analyzing batches made up of DNA from several individuals simultaneously, as pseudogens may be encountered in this material, and results could wrongfully give indications of the presence of new haplogroups. In addition, high-throughput technologies will also make it possible to carry out studies of population genomics crossing phenotypes and genotypes using genome-wide QTL (quantitative trait locus) mapping. For example, Bulked Segregant Analysis (BSA) on susceptible and resistant populations crossed in the laboratory under controlled acaricidal pressures could allow the identification of mutations responsible for resistance, as has been done on *Tetranychus urticae*, a plant mite where polygenic resistance to METI (Mitochondrial Electron Transport Inhibitors) has been demonstrated (66).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the GenBank repository. The accession number(s) are MW392987-MW393187 (mt-CO1), MW401582-MW401598 (CO1-NUMTs), MW401552-MW401581 (Tpm intron). Detailed correspondances between accession numbers, DNA region, mite individuals and sampling location can be found in **Supplementary Data Sheet 1**.

AUTHOR CONTRIBUTIONS

LR and ØØ designed the project with contribution from AG. LR conducted the analyses and wrote the first version of the manuscript. LR and NS obtained the funding. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.650546/full#supplementary-material

Supplementary Material 1 | Distribution of the groupings assigned by the authors of the studies considered here in the evolutionary history of the different CO1 haplotypes in *D. gallinae*.

Supplementary Material 2 | Amino-acid sequences translated from the CO1 haplotypes (a single copy per haplotype) of *D. gallinae*. The sequences of haplogroups H (region 1 data set; left) and *E and F (region 2 data set, right) show substantial differences from the sequences of D. gallinae s.s.* and other *Dermanyssus* species. The differences are more important between these haplogroups and the non-*gallinae* species. Sequences from the (20) dataset related to typical haplogroups of *D. gallinae s.s.* (A, B, C) show scattered differences in amino acid sequence, probably due to the concomitant amplification of a NUMT and a true mitochondrial sequence. The CO21-22 and JOW haplogroups also show some differences in the composition of their sequences compared to the others, but this is less marked.

Supplementary Material 3 | Heatmap of the frequencies of Co1 haplogroups and Tpm alleles within the isolates studied by (4, 24) and in the present study, as well as diversity indices. No clear association between a mitochondrial lineage and a nuclear allele appears within *D. gallinae* s.s. (all Tpm alleles are present with substantial frequencies in any of the isolates attached to each haplogroup). On the other hand, there is a marked separation between the two wildlife populations and all other populations.

Supplementary Material 4 | Evolutionary history of Tpm alleles (nuclear DNA) recorded from *Dermanyssus* until now, ie from (2, 4) and the homozygous sequences from present data (1,026 nucleotide sequences; total of 735 positions in the final dataset). On farms, most heterozygous individuals carry two distant alleles, belonging respectively to the clades Tro 1, Tro 2, or Tro 3.

Supplementary Data Sheet 1 | Sampling information associated with accession numbers of DNA sequences were obtained in the present study.

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