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Current knowledge on camelids infectious and parasitic diseases

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Current knowledge on camelids infectious and parasitic diseases

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Editorial: Current knowledge on camelids infectious and parasitic diseases

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Editorial on the Research Topic
Current knowledge on camelids infectious and parasitic diseases

We invited camelid scientists across the globe to submit their research on camelid diseases to highlight the importance of diseases of camelids, including Old World camels (OWC; one-humped dromedary and two-humped Bactrian camels) and New World camels (NWC; llama, alpaca, guanaco, vicuna) also known as South American camelids (SAC). We welcomed submissions in the broad subject area of endo- and ectoparasitic, bacterial, viral, and fungal infections of old and new world camelids. Finally, we accepted 10 articles on dromedaries ($n = 7$), Bactrians ($n = 2$), and NWCs ($n = 1$) written by 83 authors from 11 countries.

Tick infestation of camels is a significant challenge, impacting not only their productivity and wellbeing but also several pathogens they may transmit, some of which are zoonotic. Although chemical acaricides have been the primary means of controlling ticks, there is growing interest in developing environment-friendly herb-based acaricides. In this Research Topic, a groundbreaking study by Gareh et al. explored the acaricidal potential of neem seed extracts (*Azadirachta indica*) on the camel tick *Hyalomma dromedarii*, which is considered the most prevalent tick-infesting dromedaries. The hexane extract was the most effective, showing 100% tick mortality within 1 day at a 20% concentration. This research points to a natural alternative for tick control and emphasizes the economic viability of using neem seed extracts.

Intestinal protozoan parasites of dromedary camels have yet to be extensively studied to clarify their occurrence, diversity, and zoonotic potential since most of the research on the subject has relied on microscopic examination of fecal material. In this Research Topic, Elmahallawy et al. investigated PCR positivity of camels' fecal specimens in Egypt for *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bieneusi*. Although *E. bieneusi* was not detected, identification of zoonotic *C. parvum*, *C. bovis* and *Giardia duodenalis* implies the role of camels as sources of oo (cyst)/spore contamination in the environment. In this article global occurrence and genetic diversity of *Cryptosporidium* spp., *Giardia duodenalis*, and *E. bieneusi* reported in OWCs is summarized in a table.

The protozoan parasite *Neospora caninum* which infects many species of warm-blooded animals is a major cause of bovine abortion worldwide. Although there is lots of information about neosporosis of dromedaries, there is shortage of knowledge regarding Bactrians exposed to *N. caninum*. In the first epidemiological study on neosporosis in camels in China, Qi et al. examined serum samples from nine animal species including *Camelus bactrianus* by indirect ELISAs detecting *N. caninum*-specific IgG and IgM antibodies. The findings suggested significant exposure, emphasizing the need for further exploration into the role of different animals in the epidemiology of this ubiquitous parasite.

Eimeriosis is an economically important parasitic disease in all camelid species. In an article from Mongolia, Khatanbaatar et al. examined 536 fresh fecal samples from Bactrians to identify *Eimeria* parasites diversity, then screened the genetic diversity in a functional important immune response gene of the major histocompatibility complex (MHC). This research not only identifies *E. cameli*, *E. rajasthani* and *E. dromedarii* but also delves into the immunogenetic response of infected and non-infected camels. Understanding the host-parasite interactions is crucial for developing effective strategies against this parasitic disease.

Mastitis, an inflammatory condition of the mammary gland, one of the most significant infectious diseases affecting camels, causes substantial financial losses since it lowers the quantity and quality of milk produced. Bacterial infections are a common cause of mastitis in these animals. The primary pathogens involved in camelid mastitis include *Staphylococcus aureus*, *Streptococcus* species, and *Escherichia coli*. An efficient immune response to mastitis pathogens depends on the mammary glands' resistant cell makeup and function being in balance. In this Research Topic, mastitis is explored at the cellular level by Alhafiz et al. The study reveals changes in immune cell composition and function in camels with subclinical mastitis. These insights into the mammary gland's immune response contribute to a better understanding of host-pathogen interactions.

Globally, tuberculosis (TB) is a significant public health concern, particularly in developing countries with tropical climates. However, there are very few reports of congenital tuberculosis in people and animals. Narnaware et al. reported congenital TB caused by *M. tuberculosis* in a dromedary camel fetus with a possible vertical transmission. Authors suggested regular screening of camels for mycobacterial infection to minimize the risk associated with the spread of TB in endemic areas.

The study of coronaviruses has grown significantly in recent years. MERS-CoV replicates in various cell types, and quick development has been made of assays for its growth and quantification. However, only a few viral isolates with complete characterization are available for investigation. Middle East respiratory syndrome coronavirus (MERS-CoV) has been a focus of global health concern. Khalafalla et al. isolated and genetically characterized MERS-CoV from Dromedary Camels in the UAE in this Research Topic. The isolates contained several amino acid substitutions, and the analysis further identified a recombination event in one of the reported sequences. The findings underscore the importance of continuous monitoring and characterization of MERS-CoV for effective control measures.

Camels have been long implicated in transmitting various zoonotic diseases but the discovery of MERS-CoV sparked interest in camels as reservoirs of zoonotic pathogens. Khalafalla reviewed the literature on zoonotic diseases transmitted from camels, focusing on those with epidemiological or molecular evidence of transmission from camels to humans. This comprehensive review highlights diseases such as MERS, brucellosis, and anthrax, emphasizing surveillance, preventive measures, and a one-health approach to mitigate risks.

Apart from the pathogens and diseases, one of the most critical aspects of infection prevention and animal disease control is livestock farmers' Knowledge, Attitude and Practices (KAP). In the heart of camel-keeping regions in Kenya, the KAP survey by Othieno et al. sheds light on the challenges faced by camel herders. Respiratory diseases, indiscriminate drug use, and various constraints are identified, emphasizing the need for targeted interventions and heightened awareness. According to the authors, watering points, grazing areas, and marketing points are the primary areas for congregating camels and have a significant potential for disease spread.

Although the husbandry of South American camelids (SAC) is becoming more popular and different diseases and conditions cause emaciation, there is lack of a standardized guideline for the body condition score (BCS) in llamas or alpacas. Wagener et al. evaluated comparability of BCS assessment of six examiners including veterinarian, veterinary student and animal keeper given to 20 llamas and nine alpacas in Germany. The findings highlight the reliability of palpation of the lumbar vertebrae as a method for determining nutritional status in llamas and alpacas and that reproducibility increases with training and experience.

In conclusion, these articles collectively contribute to our understanding of camel health, revealing insights into camel health from the molecular world of parasites to the broader context of zoonotic risks. The findings underscore the importance of comprehensive research, surveillance, and awareness to ensure the wellbeing of camels and mitigate potential health risks to humans.

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Milk Immune Cell Composition in Dromedary Camels With Subclinical Mastitis

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Mastitis represents one of the most important infectious diseases in camels with heavy economic losses due to reduced milk quantity and quality. Balanced immune cell composition and function in the mammary gland are essential for effective immune response to mastitis pathogens. The objective of the present study was to characterize the cellular immune response to subclinical mastitis in the mammary gland of dromedary camels. Therefore, immunostaining and flow cytometry were used to compare the cellular composition, leukocyte phenotype, and cell viability in camel milk from healthy she-camels ($n = 8$) and she-camels with subclinical mastitis (SCM; $n = 6$). In addition, the *ex vivo* phagocytic activity of milk phagocytes was compared between healthy and affected animals. The health status of the mammary gland was evaluated based on the California Mastitis Test (CMT) score. SCM (CMT score of ≥3 in the absence of clinical signs of mastitis) was found in six of the 56 sampled quarters (10.7 %) with only one affected quarter per animal. In comparison to milk from healthy camels, milk from SCM animals showed higher somatic cell count (SCC), higher numbers of CD45+ leukocytes with an expanded fraction of CD172a+ myeloid cells. Within the myeloid cell population, there was an increase in the percentage of granulocytes ($CD172a^{+}CD14^{low}$) with a decreased percentage of macrophages ($CD172a^{+}CD14^{high}$) in milk from affected animals compared to healthy animals. The decrease in lymphoid cells in SCM milk was mainly due to the decreased fraction of CD4+ helper T cells. Camel SCM was also associated with a stimulated phenotype, increased cell viability, and enhanced phagocytic activity of the milk phagocytes, macrophages and granulocytes. Collectively, the present study identified significant changes in SCC, leukocyte count, phenotype, viability, and function in association with subclinical mastitis in camels. The results of the present study support a better understanding of host-pathogen interaction mechanisms in the camel mammary gland.

Keywords: dromedary camel (Camelus dromedarius), subclinical mastitis (SCM), immune cells, flow cytometry, phagocytosis, bacteria

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INTRODUCTION

Dromedary camels are well-adapted animals to the harsh environment of semiarid and arid zones with the ability to produce milk of valuable quantity (1). The increased reports on the nutritional and health-promoting properties of camel milk resulted in a currently raised interest in camel milk with growing market demand (2, 3).

Like other dairy animals, camels may be affected by all types of mastitis (4–7). Mastitis represents an inflammatory disease of the mammary gland mainly caused by bacterial pathogens. Unlike the clinical form of the disease, subclinical mastitis is difficult to be detected, because it does not cause any visible changes in milk or udder appearance, like swelling and redness (8). In addition to its effects on animal health and welfare, subclinical mastitis is associated with huge economic losses due to the reduced milk yield and quality and high treatment costs. Moreover, it is a public health concern for camel milk consumers (9).

The characterization of the innate and adaptive immune response of the mammary gland to invading pathogens is essential for the prevention and control of mastitis (10). In addition to milk immunoglobulins and other humoral immune factors, the mammary gland is equipped with several innate and adaptive immune cells that orchestrate the immune response to mastitis pathogens (11–14). The cellular content of the mammary gland secretions, which is called the somatic cell count (SCC), consists of a complex network of cells including neutrophils, macrophages, lymphocytes, and epithelial cells (15, 16). The contribution of the different cell types to the cellular compartment of milk differs according to species. While macrophages are the predominant cells in healthy human and bovine milk (17, 18), the majority of cells in sow and goat milk have been identified as milk granulocytes (19–21). The assessment of the cellular composition of milk, including the absolute counting of somatic cells and the differential proportions of immune cell subpopulations is widely accepted as a valuable tool for the evaluation of the health status of the mammary gland (22–24). For milk samples from healthy camels, a broad SCC range has been reported in the literature (25) with SCC values above 125×10^3 cells/ml milk being indicative of mastitis in camel (8, 25).

In contrast to the microscopic evaluation of the cellular composition of milk, which depends on the subjective evaluation of low numbers of milk cells (26, 27), flow cytometry has the advantage of measuring high cell numbers within a short time, maximizing repeatability of test results. Flow cytometric protocols for the analysis of milk cell composition and viability have been described for several species including cattle (28), goats (19, 20), sheep (29), pigs (21), and humans (30).

In the dromedary camel, several cell surface markers have been recently identified and involved in phenotypic and functional studies. Cluster of differentiation (CD) 45 is a pan-leukocyte marker glycoprotein with tyrosine phosphatase activity involved in the maturation, activation, and differentiation of several immune cells. The hyaluronan receptor CD44 is a type I transmembrane glycoprotein that is expressed on all leukocytes and plays a role in cell–cell interactions and cell migration (31, 32). The surface molecules CD18 and CD11a represent the alpha (α) and beta (β) chains that dimerize to form the adhesion molecule lymphocyte function antigen-1 (LFA-1) (33–35). The signal-regulatory protein alpha (SIRPa; CD172a) is a myeloid cell marker with a regulatory role in several functional activities of myeloid cells (36, 37). CD14, which is mainly expressed on monocytes and macrophages, plays an essential role in the recognition of LPS during infections with Gram-negative bacteria (38). The major histocompatibility complex (MHC) class II, an antigen-presentation receptor, and the scavenger receptor CD163 are commonly used for the analysis of the functional subtype of macrophages (39). CD4 and workshop cluster 1 (WC1) antigen are cell markers that identify helper ab T cells and gd T cells, respectively (35).

Milk phagocytes, including macrophages and neutrophils, are the primary effector cells of the mammary gland innate immune system with a key role during mammary gland infections (17). They contribute to the early elimination of bacterial pathogens by several antimicrobial functions, including phagocytosis, production of reactive oxygen and nitrogen species, and formation of extracellular traps (40).

The mammary gland immune response associated with subclinical mastitis pathogens in camels is still somewhat under-researched in comparison with other dairy animals. The present study was therefore aimed at the comparison of the composition, phenotype, viability, and antimicrobial functions of milk leukocytes from healthy camels and camels with subclinical mastitis.

MATERIALS AND METHODS

Animals

Investigations were conducted on milk samples collected from 14 clinically healthy dromedary she-camels during their first 2 months of lactation. The animals were reared on a private camel farm in Al-Ahsa region in eastern Saudi Arabia. All camels were from Al-majaheem camel breed and in their third and fourth lactations. All animal procedures were approved by the Ethics Committee of King Faisal University (Approval No. KFU-REC-2021- DEC -EA000326).

Clinical Examination and Milk Sampling

Milk samples were collected from all mammary gland quarters during the evening milking. Before sampling, the udder was palpated and checked for signs of clinical mastitis, such as heat, swelling and pain in the infected quarters, and abnormal alteration in milk color and consistency (41). After discarding the first milk jets, teat ends were cleaned and disinfected and about 10 ml milk were collected into sterile glass tubes for the California Mastitis Test (CMT), microbiological analysis, and SCC. Another 100 ml of milk were milked into sterile glass bottles for cell separation and flow cytometry. The health status of the animals was classified as healthy or mastitic based on the results of the CMT (41, 42). For this, 3 ml of each quarter milk were added to an equal amount of CMT fluid and the mixture was rotated by circular movement. The reactions were graded according to the Scandinavian scoring system as previously described (41). A score of 1 was given if there was no visible thickening of the mixture; score 2 represented slight slime which tends to disappear with continued swirling; score 3 indicated distinct slime but without gel formation; score 4 represented the immediate formation of gel that moves as a mass during swirling; score 5 was given if the gel developed a convex surface and adhered to the bottom of the paddle. Animals with milk samples of a test score equal to or more than 3 in the absence of clinical

signs of mastitis were classified as subclinical mastitis animals. For healthy she-camels ($n = 8$ animals) with a test score of <3 and no clinical signs of mastitis, pooled composite milk samples representing all four quarters were prepared for flow cytometry. In the affected group ($n = 6$ animals), only milk samples collected from the affected quarters were further processed for SCC and flow cytometry. Collected milk samples were kept in a cool box and were further processed in the lab within 4 h from the time of collection.

Somatic Cell Count

Milk SCC was performed after fat globule removal by the spin-wash method (43). Milk samples (500 µl) were diluted with 500 µl PBS in a 1.5 ml tube and the diluted samples were centrifuged at 1,000×g for 2 min. The upper cream layer was removed using a cotton swab and the remaining skim layer was poured off. For the second wash, 1 ml PBS was added to the tube without resuspending the pellet. The washing step was repeated twice. After the final wash, the cell pellet was resuspended in 500 µl PBS by gently pipetting up and down. The washed cell suspension (100 µl) was stained to an equal volume of Turk solution, which stains the cell nuclei blue, and the SCC was performed using Neubauer counter and light microscopy (44).

Bacteriological Analysis

For bacteriological analysis, 10 µl of milk were streaked on blood agar and MacConkey agar plates, and were incubated for 24-48 h at 37°C. The plates were then examined for growth colony morphology. Individual colonies were picked for microscopic identification using Gram staining (45). Briefly, thin smears were prepared from the plate cultures, allowed to air dry, and then fixed with heat. Smears were covered with crystal violet solution for 1 min followed by gentle rinsing with water. After that, the smears were covered with Gram iodine solution for 1 min followed by rinsing with water. After that, decolorizer solution was added to the smears for 20 s. Finally, counter-staining with safranin solution was performed for 1 min followed by rinsing with water. The smears were examined microscopically at 1,000×magnification with oil immersion. The bacterial species were identified based on the shape, arrangement and gram reaction of the organisms as previously described (46).

Cell Separation

Eight milk samples collected from eight healthy animals (each representing four quarter milk samples) and six milk samples collected from affected quarters of six affected animals were used for cell separation and flow cytometry. Separation of milk cells was performed according to a method previously described for caprine milk (47) with some modifications. Briefly, milk samples were diluted with cold PBS (25 ml milk and 25 ml PBS) in conical 50 ml polypropylene tubes and the tubes were centrifuged at 800×g and 4°C for 20 min without brake. After removing the fat layer using a spatula, the supernatant was discarded. The cell pellet was resuspended with 30 ml cold PBS and washed twice at 600×g and 4°C for 10 min. For parallel staining of blood leukocytes, leukocytes were separated from one EDTA blood sample collected from a healthy dromedary camel as previously described (48). Concisely, 1 ml blood was incubated with 5 ml distilled water for 20 s followed by the addition of 2× PBS to restore tonicity. After centrifugation (1,000×g, 15 min, 10°C, without brake), the supernatant was discarded and the washing step was repeated twice (at 500×g and 250×g for 10 min) until complete removal of red blood cells. Blood and milk cell pellets were suspended in 1 ml cell staining buffer (PBS containing 5 g/l BSA, 100 mg/l NaN3) at concentrations of 5×10^6 cells/ml. Cell viability was determined after incubating the cells with the nucleic acid stain propidium iodide (PI; 2 µg/ml, BD Biosciences, Germany).

Antibodies

The monoclonal and polyclonal antibodies used are shown in **Table 1** (44, 48, 49). Leukocytes were identified using a monoclonal mouse antibody against lama pan-leukocyte marker CD45 (clone LT12A). A monoclonal antibody to the myeloid cell marker signal-regulatory protein alpha (SIRPα), also called CD172a (clone DH59b), was used to differentiate myeloid cells from lymphoid cells. Macrophages and granulocytes were differentiated based on their differential expression of the lipopolysaccharide receptor CD14, which was detected using a mouse anti CD14 monoclonal antibody (clone Tuk4). The antibodies used for helper T cells and gamma delta T cells were mouse anti bovine CD4 (clone GC50A1), and WC1 (clone BAQ128A) antibodies. The monoclonal antibodies to major histocompatibility complex (MHC) class II (clone TH81A5) and the scavenger receptor CD163 (clone LND68A) were used for the analysis of macrophages phenotype. Furthermore, the monoclonal antibodies to CD18 (clone 6.7), CD11a (clone HUH73A), and CD44 (LT41A) were used to measure the expression of selected cell adhesion molecules. All antibodies were tested for reactivity against camel leukocytes in previous studies (35, 50–53). Secondary antibodies to mouse primary antibodies were goat anti-mouse IgM conjugated with Allophycocyanin (APC), goat anti-mouse IgG1 conjugated with fluorescein isothiocyanate (FITC), and goat anti-mouse IgG2a conjugated with phycoerythrin (PE).

Cell Labeling and Flow Cytometry

Cell labeling was performed in a three steps staining procedure in a round-bottomed 96-well microtiter plate as previously described (47). For this, 100 µl of the leukocyte suspension (5×10^5 leukocytes per well) of each sample were pipetted into the well of a microtiter plate. After short centrifugation of the plate for 3 min at 300×g and 4°C, the supernatant was discarded and the primary antibodies (**Table 1**) diluted in staining buffer were added to the wells. After 15 min of incubation at 4°C, the cell suspension was washed with staining buffer for 3 min at 300×g and 4°C. In the second staining step, fluorochrome-conjugated secondary antibodies (**Table 1**) to mouse primary antibodies were added to the wells and the plate was incubated for an additional 15 min at 4°C in the dark. Subsequently, the cells were washed twice with staining buffer for 3 min at 300×g and 4°C. In the final staining step, an anti-CD14 monoclonal antibody conjugated with APC was added to selected wells followed by a 15 min incubation at 4°C in the dark. Finally, the cells were washed twice

TABLE 1 | List of monoclonal and polyclonal antibodies.

Antigen	Antibody clone	Labeling	Source	Isotype
CD14	CAM36A	-	Kingfisher	Mouse IgG1
CD14	Tuk4	APC	Thermofisher	Mouse IgG2a
MHCII	TH81A5	-	Kingfisher	Mouse IgG2a
CD172a	DH59b		Kingfisher	Mouse IgG1
CD163	LND68A	-	Kingfisher	Mouse IgG1
CD4	GC50A1	-	VMRD,	Mouse IgM
WC1	BAQ128A	-	VMRD,	Mouse IgG1
CD11a	HUH73A	-	Kingfisher	Mouse IgG1
CD18	6.7	FITC	BD	Mouse IgG2a
CD44	LT41A	-	Kingfisher	Mouse IgG2a
CD45	LT12A	-	Kingfisher	Mouse IgG2a
Mouse IgM	poly	APC	Thermofisher	Goat IgG
Mouse IgG1	poly	FITC	Thermofisher	Goat IgG
Mouse IgG2a	poly	PE	Thermofisher	Goat IgG

MHC, Major Histocompatibility Complex; WC1, workshopcluster 1; APC, Allophycocyanin; FITC, Fluorescein isothiocyanate; PE, Phycoerythrin; poly, polyclonal.

with staining buffer for 3 min at 300×g and 4°C and resuspended in 150 µl staining buffer for flow cytometry. Staining with only antibody isotype controls was included. Labeled cells were analyzed on a FACSCalibur (BD Biosciences) by the acquisition of at least 100 000 total cells. Collected flow cytometric data were analyzed using the FCS Express Software (V3; BD Biosciences).

Phagocytosis Activity of Milk Phagocytes

Bacterial phagocytosis by milk phagocytes was performed using heat-killed *staphylococcus aureus* (*S. aureus*) bacteria (Pansorbin, Calbiochem, Merck, Nottingham, UK) labeled with fluoresceinisothiocyanate (FITC, Sigma-Aldrich, St. Louis, Missouri, USA) (54). Separated milk cells (2×10^5 in 100 µl RPMI medium) were incubated with *S. aureus-FITC* (50 bacteria/cell) for 45 min at 37°C in 96 well plates. After incubation, the plate was washed in RPMI medium (300×g for 3 min) and the cells were resuspended in 150 µl of staining buffer and analyzed by flow cytometry. After the identification of the phagocyte population including granulocytes and macrophages, the percentage of phagocytosis-positive cells, as well as their mean green fluorescence intensity (MFI), were calculated.

Statistical Analyses

Data were processed with the Microsoft office Excel® program (version 2016 Microsoft) and statistical analysis was performed using the software program Prism (GraphPad software version 5, GraphPad Software, San Diego, USA). The Kolmogorov-Smirnov test (with the Dallal-Wilkinson-Lilliefor *P*-value) was performed to check the normal distribution of data. For normal-distributed data, the unpaired student's *t*-test was used to compare the mean of the two groups. For the data that failed to pass the normality test, the non-parametric Mann-Whitney test was used to compare the means. The comparison between milk granulocytes, macrophages, and lymphocytes regarding their phenotype was performed using a one-factorial analysis of variance (ANOVA) with Bonferroni's Multiple Comparison Test. The results for each analyzed parameter were presented graphically as means ± standard error of the mean (SEM). Results were considered statistically significant if the *p*-value was <0.05.

RESULTS

Flow Cytometric Identification of Camel Milk Leukocyte Subpopulations

Using monoclonal antibodies to the leukocyte antigens CD172a and CD14, camel granulocytes, macrophages, and lymphocytes were identified in camel milk samples by flow cytometry. To confirm the expression pattern of the cell surface molecules, blood leukocytes were separated from one animal and were stained with the same combination of monoclonal antibodies (**Figure 1**). Camel milk granulocytes were identified based on their positive staining with the myeloid marker CD172a and low staining with CD14 ($CD172a^{+}CD14^{low}$), while milk macrophages expressed both markers in high levels ($CD172a^{+}CD14^{high}$). On the other hand, milk lymphocytes were identified based on their negative staining with the myeloid marker CD172a and the monocytic marker CD14 ($CD172a^{-}CD14^{-}$).

The Immunophenotype of Camel Milk Leukocyte Subpopulations

Milk macrophages showed the highest abundance ($p < 0.05$) of the myeloid marker CD172a (SIRP-alpha), the lipopolysaccharide receptor CD14, the scavenger receptor CD163, the antigen presentation receptor major histocompatibility complex (MHC) II molecules, the cell adhesion molecules CD18 (integrin beta chain), and CD11a (the alpha chain of the lymphocyte function-associated antigen 1; LFA-1) when compared to granulocytes and lymphocytes (**Figure 2A**). While granulocytes displayed an intermediate level of CD172a, CD14, CD163, MHCII, CD18, and CD11a, lymphocytes showed the lowest abundance of all those molecules, in comparison to granulocytes and macrophages (**Figure 2A**).

The analysis of light scatter characteristics of milk leukocyte subpopulations revealed similar ($p > 0.05$) cell size (as measured by the forward scatter; FSC) and granularity (as measured by side scatter; SSC) for milk granulocytes and macrophages ($FSC^{high}SSC^{high}$), while milk lymphocytes were identified as smaller cells with lower granularity ($FSC^{low}SSC^{low}$), in comparison to granulocytes and macrophages ($p < 0.05$) (**Figure 2B**).

Subclinical Mastitis

Subclinical mastitis (SCM) was diagnosed based on the California Mastitis Test (CMT) score and the absence of signs of clinical mastitis. SCM (CMT score of ≥3 in the absence of clinical signs of mastitis) was found in six of the 56 sampled quarters (10.7%). All affected camels had only 1 quarter with SCM. The bacteriological analysis identified

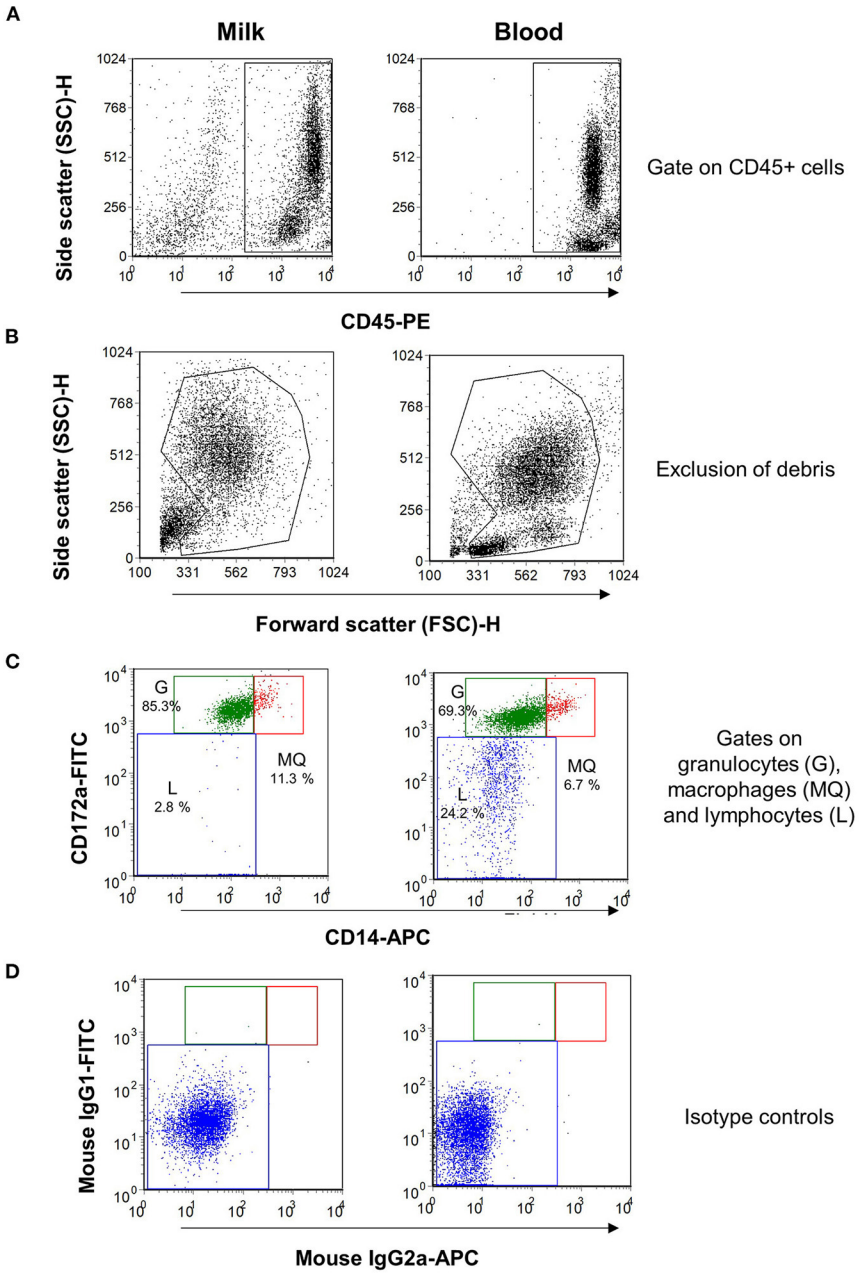

FIGURE 1 | Flow cytometric analysis of leukocytes in milk and blood from dromedary camel. Separated milk and blood leukocytes were labeled with monoclonal antibodies to CD45, CD172a, and CD14, and stained cells were analyzed by flow cytometry. **(A)** Using an SSC/CD45 gate, milk, and blood leukocytes were identified as CD45-positive cells. **(B)** Cell debris were excluded in an FSC-H/SSC-H dot plot. **(C)** Milk and blood granulocytes (G), macrophages (MQ), and lymphocytes (L) were identified based on their staining with monoclonal antibodies to CD172a, and CD14. **(D)** Cells stained with mouse IgG1 and IgM isotype controls.

bacterial cultures in all milk samples collected from the six SCM quarters and only in one sample collected from healthy animals. While two milk samples yielded single bacterial cultures with *staphylococcus* or *streptococcus* species, the other five samples yielded mixed bacterial cultures with *streptococcus* and *staphylococcus* (three samples) or *streptococcus* and coliform bacteria (two samples).

Somatic Cell Count, Total and Differential Leukocyte Composition in Milk Samples From Healthy Camels and Camels With Subclinical Mastitis

The SCC, the fraction of milk leukocytes (CD45+ cells), and the differential leukocyte composition were compared between the healthy and SCM animals. The SCC was significantly ($p =$

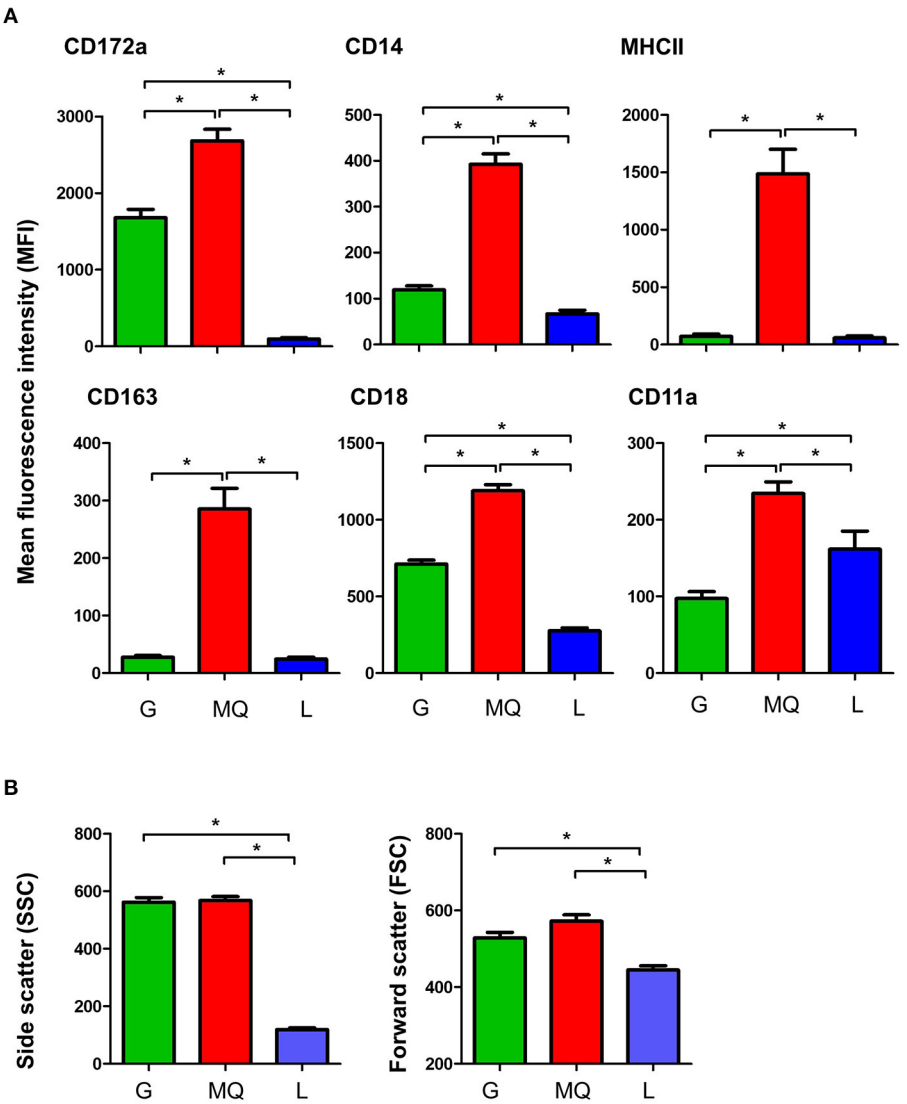

FIGURE 2 | (A) The expression levels of the cell surface antigens, CD172a, CD14, MHCII, CD163, CD18, and CD11a on milk granulocyte (G), macrophages (MQ), and lymphocytes (L) as measured by flow cytometry. **(B)** Forward and side scatter characteristics of milk granulocytes (G), macrophages (MQ), and lymphocytes (L) as measured by flow cytometry. *indicates statistically-significant difference between the means.

0.001) higher in milk samples from SCM camels (418.3 $\times$ 10^3 cell/ ml) than healthy animals (103.8 $\times$ 10^3 cell/ml) (**Figure 3A**). Milk samples with SCM contained higher percentages (p = 0.001) of total leukocytes (90.3 $\pm$ 3.1% of total cells) than healthy (48.5 $\pm$ 9.3% of total cells) milk samples (**Figure 3B**). The fraction of myeloid cells within the leukocyte population was also significantly elevated (p = 0.01) in SCM milk samples (97.8 $\pm$ 0.5% of leukocytes) compared to healthy milk samples (93.5 $\pm$ 1.3% of leukocytes) (**Figure 3C**), while the fraction of lymphoid cells was significantly (p = 0.01) lower in SCM than in healthy milk samples (**Figure 3D**). Similarly, the fraction of milk granulocytes was significantly (p = 0.02) expanded in SCM samples (79.1 $\pm$ 2.7% of leukocytes vs. 67.8 $\pm$ 3.7% of leukocytes in healthy milk) (**Figure 3E**), while the fraction of macrophages was decreased (16.2 $\pm$ 3.4% of leukocytes vs. 24.7 $\pm$ 3.6% of leukocytes in healthy milk; p = 0.04) (**Figure 3F**).

Impact of Subclinical Mastitis on Milk Leukocyte Viability

The percentage of viable, PI-negative (**Figure 4A**) milk leukocytes was significantly (p = 0.01) higher in milk samples from SCM animals (91.9 $\pm$ 1.9% of CD45+ cells) than healthy animals (85.8 $\pm$ 1.3% of CD45+ cells). In milk from SCM animals, the myeloid cell population contained a higher (p < 0.05) percentage of viable cells (91.9 $\pm$ 1.7% vs. 77.0 $\pm$ 3.3% of CD172a+ myeloid cells), while the percentage of viable cells under lymphoid cells was significantly lower (p < 0.05) in SCM

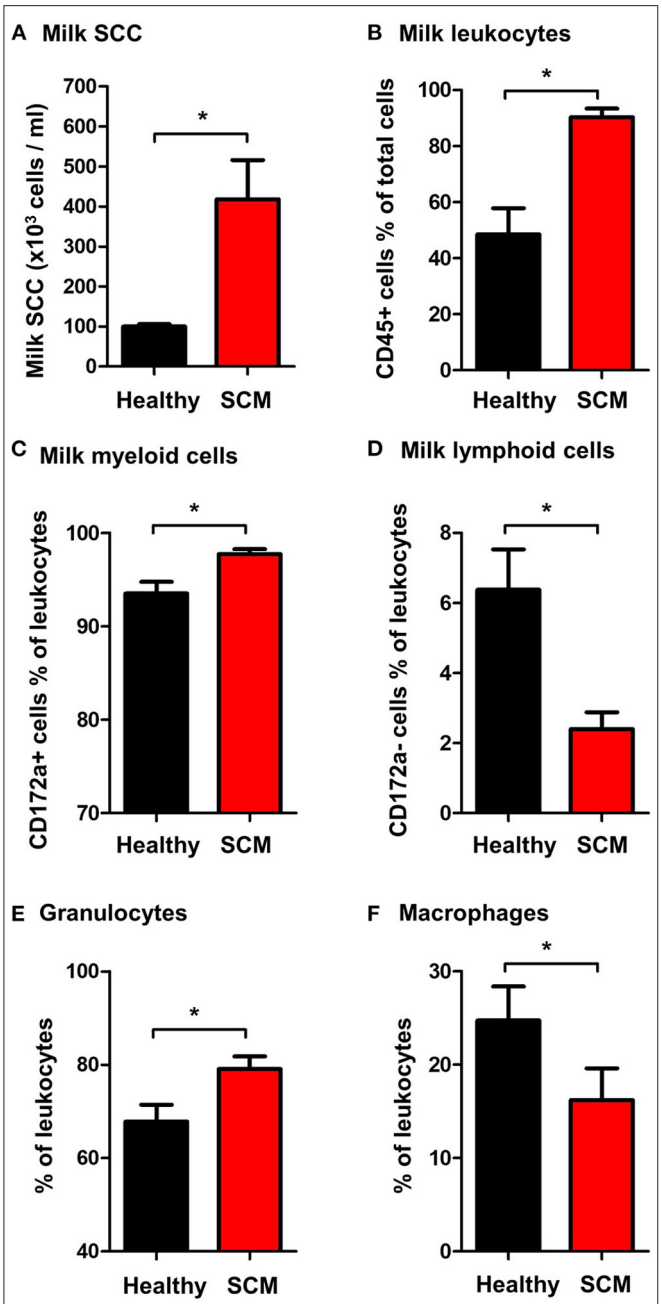

FIGURE 3 | (A) Somatic cell count (SCC) in milk samples from healthy camels and camels with subclinical mastitis (SCM). Milk SCC was counted using the direct microscopic method after fat globule removal by a spin-wash followed by staining with Turk solution and microscopic counting on a Neubauer cell counter. The percentage of **(B)** leukocytes (CD45+ cells % of total milk cells), **(C)** myeloid cells (CD172a+ cells % of total leukocytes), **(D)** lymphoid cells (CD172a- cells % of total leukocytes), **(E)** granulocytes (CD172a+ CD14low cells % of total leukocytes), and **(F)** macrophages (CD172a+ CD14high cells % of total leukocytes) in milk samples from healthy and SCM camels as identified by flow cytometry after labeling milk cells with antibodies to CD45, CD172a, and CD14. *indicates significant differences between the means with *p*-values < 0.05.

milk (85.2 ± 4.3% vs. 91.0 ± 2.2% of CD172a-lymphoid cells) compared to healthy milk (**Figure 4B**).

Milk Lymphocytes From Animals With SCM Contained a Lower Percentage of Helper T Cells

Using monoclonal antibodies to camel CD4 and WC1, the percentages of helper T cells and γδ T cells were analyzed in milk from healthy and SCM animals (**Figure 5A**). While the fraction of WC1+ γδ T cells was comparable ($p > 0.05$) between milk samples from healthy and SCM animals, the percentage of CD4+ T helper cells was significantly ($p = 0.002$) lower in SCM (7.8 ± 1.4% of lymphocytes) than healthy milk (16.3 ± 1.8% of lymphocytes) (**Figure 5B**).

Granulocytes and Macrophages Shape Change in SCM Milk

The analysis of forward scatter (FSC) and side scatter (SSC), which are indicators for cell size and granularity, respectively, revealed higher ($p < 0.05$) FSC values for granulocytes and macrophages from SCM milk compared to healthy milk (**Figure 6A**). Only for granulocytes, the SSC values were lower ($p < 0.05$) in SCM milk compared to healthy milk (**Figure 6B**).

Impact of SCM on the Phenotype of Milk Macrophages

The comparison between macrophages from SCM and healthy milk regarding their expression levels of several cell surface molecules revealed significant changes in their phenotype. While the abundance of cell surface CD172a, CD163, and CD18 did not differ ($p < 0.05$) between SCM and healthy milk samples, macrophages from SCM milk showed higher levels of MHCII but lower levels of CD14 and CD11a when compared ($p < 0.05$) to macrophages from healthy milk (**Figure 7**).

Impact of SCM on the Antimicrobial Function of Milk Phagocytes

The antimicrobial function of milk phagocytes (granulocytes and macrophages) was analyzed by the evaluation of bacterial phagocytosis by flow cytometry (**Figure 8A**). The percentage of phagocytosis-positive cells was higher ($p < 0.05$) for phagocytes from SCM milk than healthy milk. The phagocytic capacity (the number of bacteria ingested by each cell as measured by MFI of phagocytosis-positive cells), however, did not differ ($p > 0.05$) between cells from SCM and healthy milk (**Figure 8B**).

DISCUSSION

The immune cell composition of the mammary gland secretions has been investigated for several animal species in health and disease (19, 20, 55–58). However, studies on the cellular composition of camel milk are limited. The present study employed flow cytometry and monoclonal antibody staining to investigate the differential composition, phenotype, vitality, and

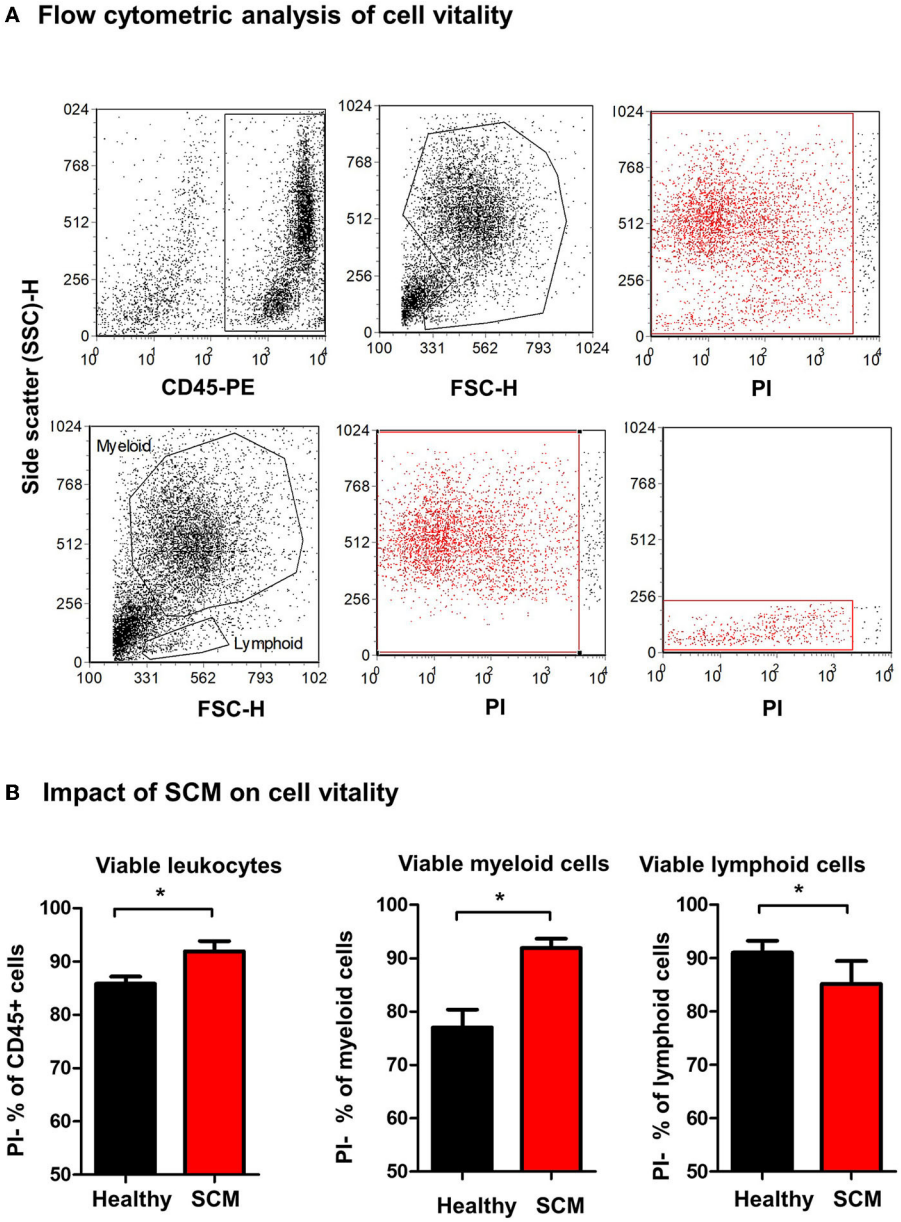

FIGURE 4 | Separated milk cells were labeled with CD45 antibodies and labeled cells were loaded with the nucleic acid stain propidium iodide and analyzed by flow cytometry. **(A)** After gating on leukocytes (CD45 + cells) and the exclusion of cell debris in a SSC-H against FSC-H dot plot, milk phagocytes and lymphocytes were gated based on their FSC and SSC properties. **(B)** The percentage of viable PI-negative cells were calculated in a SSC-H against FL-3 dot plot and the values were presented for total leukocytes, myeloid cells, and lymphoid cells, and presented graphically. *indicates significant differences between the means with p-values < 0.05.

some functional aspects of milk leukocytes in clinically healthy camels. The evaluation of the health status of the mammary gland and the classification of the camels was based on the results of the California Mastitis Test (CMT) with a test score of $\geq$3 in the absence of signs of clinical mastitis being indicative for subclinical mastitis (41).

The present study divided camel milk leukocytes based on their differential expression of the cell surface molecules CD172a and CD14 into a dominant $CD172a^{+}/CD14^{low}/SSC^{high}$ granulocyte population followed by a smaller fraction of $CD172a^{+}/CD14l^{high}/SSC^{high}$ macrophages and a minor $CD172a^{-}/CD14^{-}/SSC^{low}$ lymphocytes population. The higher proportion of granulocytes in camel milk, compared to other species like bovine (17, 28), could be a result of their dominance in peripheral blood (49). The results of the present study, however, largely correspond to data reported for goats regarding the differential composition of milk cells (19, 20). The expression pattern of the cell surface antigens, CD172a, CD14, CD163, MHCII, CD11a, and CD18 on milk granulocytes and macrophages indicates similarities with the immunophenotype

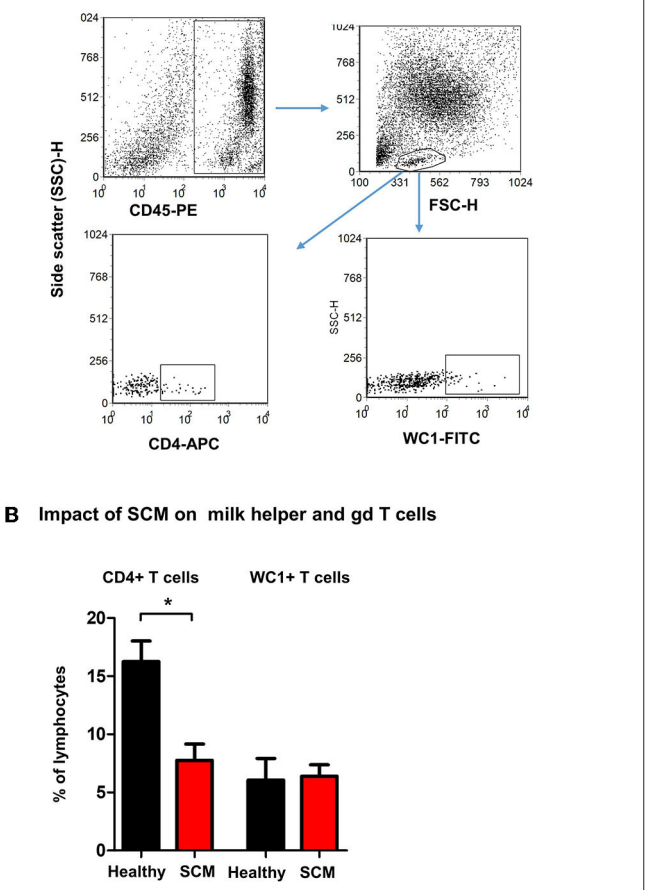

FIGURE 5 | Flow cytometric analysis of milk lymphocyte subsets. Separated milk cells were labeled with monoclonal antibodies to CD45, CD4, and WC1 and analyzed by flow cytometry. **(A)** After setting a gate on milk leukocytes in a CD45 against SSC-H dot plot, lymphocytes were identified based on their SSC and FSC properties. Milk helper T cells and gd T cells were identified within the lymphocyte population based on their staining with anti-CD4 and anti WC1 antibodies, respectively. **(B)** The percentage of T helper cells and gd T cells within the milk lymphocyte population were calculated for healthy and SCM camels and presented graphically. *indicates significant differences between the means with p-values < 0.05.

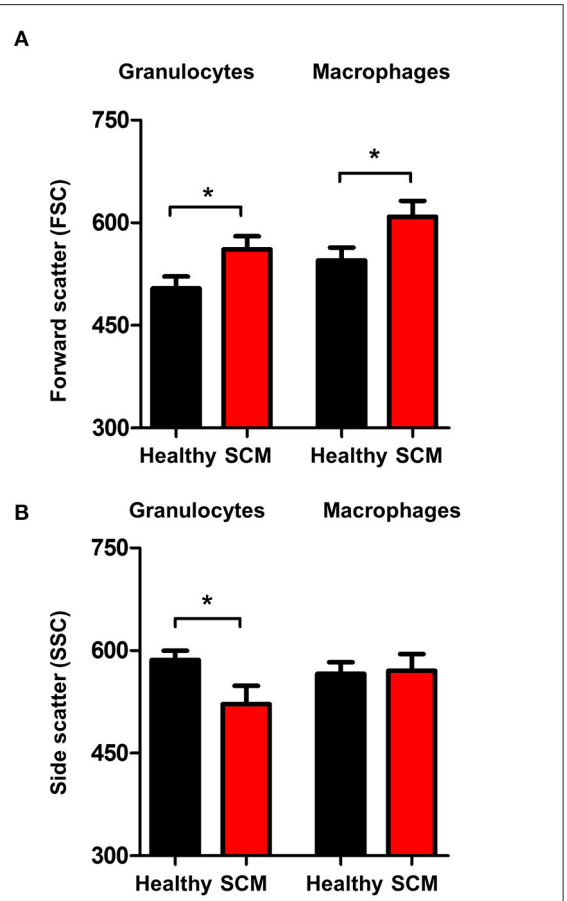

FIGURE 6 | Shape change in milk granulocytes. Milk granulocytes and macrophages were identified based on their staining with CD172a and CD14 antibodies in flow cytometry. Mean FSC **(A)** and SSC **(B)** were calculated and presented for healthy and SCM animals. *indicates significant differences between the means with p-values < 0.05.

of peripheral blood granulocytes and monocytes, respectively, with CD172a, CD18, and CD11a being highest expressed on camel monocytes/macrophages, while MHCII and CD163 being exclusively expressed on monocytes/macrophages (53).

The milk somatic cell count and the CMT are widely accepted tools for the evaluation of the mammary gland health status (17, 28). In camels, elevated SCC values were observed in milk samples from infected mammary glands with values ranging from 1×10^5 to 10×10^6 cells/ml milk (8, 25). In the present study, the four-times elevated SCC with the identification of bacterial cultures in milk samples with a CMT test score ≥ 3 confirm the results from previous reports regarding the efficiency of CMT and SCC as diagnostic tools for monitoring mammary gland infections in camels.

The two-times increase in whole milk leukocytes with more myeloid cells (CD172a+ cells) in the SCM milk suggests a significant role of the innate immune phagocytes, granulocytes and macrophages, in the immune response to bacterial infections of the camel mammary gland. The increase in the proportion of granulocytes with the decrease in macrophages in SCM milk could be a result of enhanced recruitment of blood neutrophils to the infected mammary gland.

According to reports in dairy cows with bacterial mastitis, the survival of neutrophils was higher in infected than healthy mammary glands (59). The same study identified a link between the higher viability of milk phagocytes with their enhanced antibacterial function. In the present study, the viability of granulocytes and macrophages, as well as the fraction of phagocytosis-positive cells, were higher in SCM milk compared to healthy milk, suggesting similarity in the host-pathogen interaction mechanisms in the mammary gland of cattle and camel.

The lipopolysaccharide (LPS) receptor CD14, the antigen presentation receptor MHCII, and the hemoglobin-haptoglobin

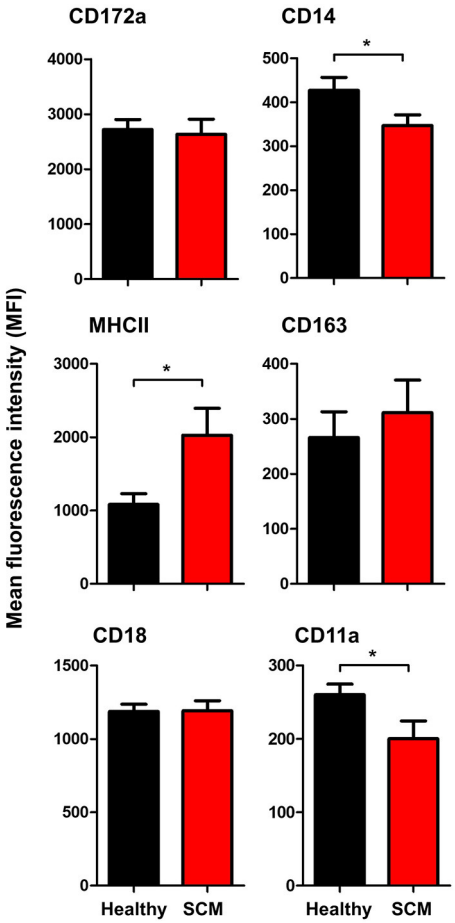

FIGURE 7 | Impact of SCM on the phenotype of milk macrophages. Separated milk cells were stained with monoclonal antibodies to CD172a, CD14, CD163, MHCII, CD18, and CD11a and stained cells were analyzed by flow cytometry. After setting a gate on milk macrophages (based on their higher expression of CD14), the mean fluorescence intensity of the monocytic cell markers and the cell adhesion molecules was calculated and presented for healthy and SCM animals. *indicates significant differences between the means with p-values < 0.05.

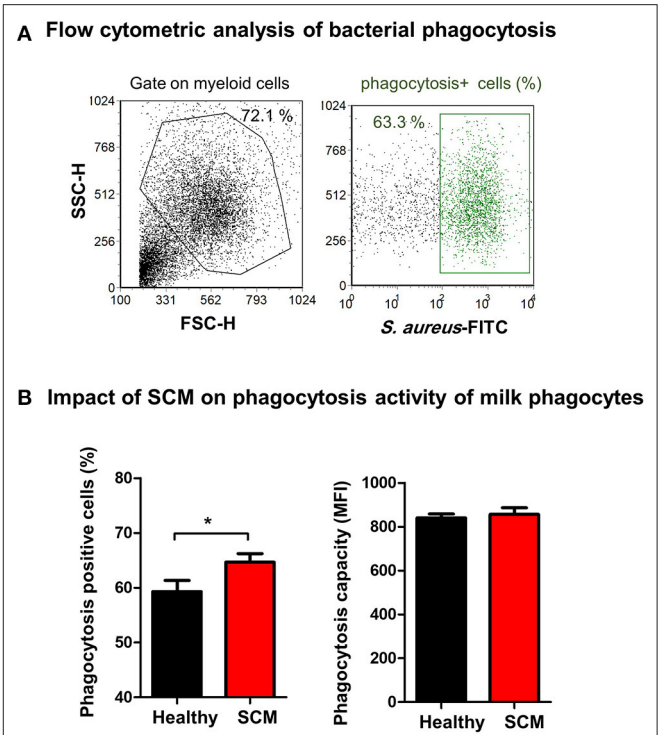

FIGURE 8 | (A) Separated milk cells were incubated with FITC-conjugated *S. aureus* and the fraction of phagocytosis-positive cells within the myeloid cell population (including granulocytes and macrophages defined based on SCG/SSC properties) was estimated by flow cytometry based on their enhanced fluorescence in the green fluorescence channel. **(B)** The percentage of phagocytosis-positive cells and their mean fluorescence intensity (MFI), which is a metric of phagocytic capacity of the cells, were calculated and presented for healthy and SCM animals.

receptor CD163 are well-established markers of monocyte and macrophage phenotype (53, 60–62). The differences in the expression levels of MHCII and CD14 on milk macrophages indicate a significant modulatory effect of subclinical mastitis on the functional type of macrophages. Reduced expression of CD14, which plays a key role in the binding of LPS, a cell-wall component of gram-negative bacteria (63–65), may have an impact on the innate recognition function of macrophages in SCM milk.

Macrophages and neutrophils are key effector innate immune cells of the mammary gland with an essential role during the early immune response to mastitis pathogens (17, 40). In the present work, the observed shape-change of granulocytes and macrophages from SCM milk with higher forward scatter values, which correlates with the cell size, and lower side scatter values of granulocytes, which is indicative of cell degranulation (66, 67), indicate the activation status of these phagocytes in the infected mammary gland. This is also supported by the higher fraction of phagocytosis-positive cells in SCM milk.

The lower percentage of helper T cells in SCM milk with no difference in the percentage of γδ T cells suggests a selective impact of bacterial mammary gland infections on T cell subpopulations. As we did not analyze all lymphocyte subsets due to the lack of specific monoclonal antibodies (49), we cannot exclude changes in other milk lymphocyte subsets like CD8+ cytotoxic T cells, B cells, or NK cells in SCM animals.

Collectively, the present study identified significant differences between healthy camels and camels with SCM regarding the cellular composition of their milk. Milk from SCM camels had higher SCC with higher fractions of total leukocytes, myeloid cells, and granulocytes, but reduced fractions of lymphoid cells and macrophages. Within the lymphoid cell population, the percentage of CD4+ T helper cells was reduced in milk from SCM camels. In addition, SCM was associated with improved cell viability and phagocytic activity of milk phagocytes. The results of the present study pave the way for the characterization of the camel immune response to mammary gland infections and support a better understanding

of host-pathogen interaction mechanisms on mucosal surfaces in camels.

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 animal study was reviewed and approved by Ethics Committee of King Faisal University.

AUTHOR CONTRIBUTIONS

GA: sample collection and sample preparation for flow cytometric analysis. FA: sample collection and flow cytometric analysis. HA: flow cytometric analysis and writing the original manuscript. JH: funding acquisition, conceptualization, flow cytometric analysis, and writing the manuscript. All authors have read and approved the final manuscript.

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Seroepidemiology of Neosporosis in Various Animals in the Qinghai-Tibetan Plateau

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Neosporosis is a worldwide infectious disease caused by intracellular parasite *Neospora caninum* that is a major pathogen of abortion in cattle and neurological disorders in other hosts. However, limited data are available on animals exposed to *N. caninum* in the Qinghai-Tibetan Plateau Area (QTPA), and little is known about whether animals in the plateau area play an important role in the epidemiology of *N. caninum*. Therefore, indirect ELISAs based on a combination of *Nc*SAG1 and *Nc*GRA7 antigens were developed to examine both *N. caninum*-specific IgG and IgM antibodies in Tibetan sheep, yak, cow, pig, cattle, horse, chicken, camel, and donkey from the QTPA in this study. The results showed that all current species present- IgG and IgM-positive animals, and that the overall seroprevalence of *N. caninum* were 18.6 (703/3,782) and 48.1% (1,820/3,782) for the IgG and IgM antibodies, respectively. Further analysis found significant differences from different altitudes in IgG in Tibetan sheep and IgM in the yak. Hence, the present serological results indicate that the tested animal populations in the QTPA are suffering from *N. caninum* infections or have become carriers of *N. caninum* antibodies. To the best of our knowledge, this is the first report on current *N. caninum*-infected animals in the QTPA, the first epidemiology of neosporosis in cow and camel in China, and the first record of *N. caninum* IgM antibodies in all the surveyed animals in China. This study provides the latest valuable data on the epidemiology of neosporosis in China and in plateau areas of the world.

Keywords: ***Neospora caninum*, IgG, IgM, animals, Qinghai-Tibetan Plateau, seroepidemiology**

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INTRODUCTION

Neosporosis is a worldwide infectious disease caused by the obligate intracellular parasite protozoan *Neospora caninum*, which is a major pathogen of abortion in cattle and reproduction problems and neurological disorders in dogs (1–6). Canines are definitive hosts shedding oocysts in the environment that play an important role in the epidemiology of neosporosis associated with *N. caninum* infections in cattle and other intermediate hosts (e.g., sheep, pigs, goats, yaks, chickens, horses, and donkeys) (1, 3, 5).

Neospora caninum infection in a large spectrum of wild and domestic animals was described in many countries, especially cattle and dogs (1, 3, 5, 7–11). Although the prevalence of neosporosis in various animal hosts has been determined in several areas in China (12–18), limited data are

available on domestic and wild animals exposed to *N. caninum* in the Qinghai-Tibetan Plateau Area (QTPA). For the epidemiology of neosporosis, serological ELISA diagnostic methods with highly specific and sensitive characteristics have been developed, and ELISAs based on specific antigens derived from *N. caninum*, especially for the case of surface antigen 1 (*Nc*SAG1) and dense granule protein 7 (*Nc*GRA7), were used to perform serological testing on parasitic infections in a large number of animal samples (19–23).

A variety of animals that are adapted to the high altitude and cold climate lives in the QTPA (24, 25), including Tibetan sheep (*Ovis aries*), yak (*Bos grunniens*), cow (*Bos taurus*), pig (*Sus domesticus*), cattle (*Bos taurus domestica*), horse (*Equus ferus caballus*), chicken (*Gallus gallusdomesticus*), camel (*Camelus bactrianus*), and donkey (*Equus asinus*). Diseases caused by infectious parasites have brought serious threats to the development of animal husbandries and human health. However, little is known whether the animals in this plateau area play an important role in the prevalence of *N. caninum*. Therefore, this present study aims to examine the serological prevalence of neosporosis using ELISAs based on the combination of recombinant SAG1 and GRA7 proteins in various animals in the QTPA. Our study should have major importance in epidemiological neosporosis in the plateau area.

MATERIALS AND METHODS

Serum Samples

A total of 3,782 serum samples were collected in nine animal species from 2,000 m above sea level to 4,897 m in two cities and six prefectures of the QTPA with geographical coordinates of 31°36′-39°19′ N and 89°35′-103°04′ E from June 2021 to February 2022 (**Table 1**) including Tibetan sheep (*O. aries*), yak (*B. grunniens*), cow (*B. taurus*), pig (*S. domesticus*), cattle (*B. taurus domestica*), horse (*E. ferus caballus*), chicken (*G. gallusdomesticus*), camel (*C. bactrianus*), and donkey (*E. asinus*). Animal serum samples were frozen and stored at −20°C until assayed. All the procedures were carried out according to the ethical guidelines of Qinghai University.

Expression and Purification of Recombinant *Nc*SAG1 and *Nc*GRA7 Proteins

The recombinant *Nc*SAG1 and *Nc*GRA7 were expressed and purified using the following protocols in this study: the SAG1 gene (GenBank: AF132217.1) and GRA7 gene (GenBank: JQ410455.1) were amplified by PCR from the cDNA of *N. caninum* parasites. Primers that included a BamH I site (underlined) in the forward primer 5′-CG *GGATCC* TCA GAA AAA TCA CCT CTA CT-3′, an EcoR I site (underlined) in the reverse primer 5′-CG *GAATTC* CGG ACC AAC ATT TTC AGC CGA CGA-3′ for *Nc*SAG1, a BamH I site (underlined) in the forward primer 5′-CG *GGATCC* GCT GGA GAC TTG GCA-3′ and an EcoR I site (underlined) in the reverse primer 5′-CG *GAATTC* CGC TAT TCG GTG TCT ACT TCC TG-3′ for *Nc*GRA7 were used. The PCR products digested with BamH I and EcoR I and inserted into the pGEX-6p-2 plasmid vector were treated with the same restriction enzymes (Roche, Switzerland). The IPTG was used to induce recombinant pGEX-6p-2-*Nc*SAG1 and pGEX-6p-2-*Nc*GRA7 expressions in *Escherichia coli* BL21 (DE3; New England BioLabs Inc., United States) at 37°C for 4 h, and then they were purified with the Glutathione Sepharose 4B beads (GE Healthcare Life Sciences) according to the manufacturer's instructions. The concentration of *Nc*SAG1 and *Nc*GRA7 proteins were measured with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, United States).

Indirect ELISAs

IgG and IgM antibodies against *N. caninum* were detected by indirect ELISA tests based on the recombinant *Nc*SAG1 and *Nc*GRA7 proteins. The 1-μg/ml recombinant proteins measured with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, United States) were diluted in a coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) to perform an indirect ELISA analysis: the current sera were diluted at 1:100, and the secondary antibodies of Rabbit Anti-Bovine IgM/HRP (bs-0327R-HRP; Bioss, China), Rabbit Anti-Bovine IgG H and L/HRP (bs-0326R-HRP; Bioss, China), Goat Anti-Horse IgM H and L/HRP (ab112879; Abcam, United Kingdom), Rabbit Anti-Horse IgG/HRP (bs-0308R-HRP; Bioss, China), Rabbit Anti-Sheep IgM/HRP (ab112763; Abcam, United Kingdom), Rabbit Anti-Sheep IgG H and L/HRP (AS023; Abclonal, China), Rabbit Anti-Pig IgG/HRP (bs-0309R-HRP; Bioss, China), HRP*Mab Pig IgM (Primadiagnostic, China), Goat Anti-Chicken IgG/HRP (bs-0310G-HRP; Bioss, China), Rabbit Anti-Chicken IgM/HRP (bs-0314R-HRP; Bioss, China), Goat Anti-Cow IgG H and L/HRP (ab102154; Abcam, United Kingdom), Sheep Anti-Cow IgM H and L/HRP (ab112752; Abcam, United Kingdom), Goat Anti-Donkey IgG H and L/HRP (ab6988; Abcam, United Kingdom), and Goat Anti-Camel IgG H and L/HRP (S003H; Nbbiolab, China) were diluted at 1:1,000−4,000. In this study, an ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] substrate was used to show the results at OD 415 nm. For the resulting judgment, the cut-off point was calculated as the mean value of OD 415 nm for standard *N. caninum*-negative sera kept in our laboratory (ten samples of each animal) plus three times the standard deviations of OD415 nm values of the negative controls: the mean X and standard deviation SD of the negative control results were calculated, and $X + 3SD$ was the cut-off value of GRA7-ELISA and SAG1-ELISA. The OD 415 value was both greater than the respective cut-off values of GRA7-ELISA and SAG1-ELISA judged as positive, that is, the samples were judged as positive animals only when both SAG1-ELISA and GRA7-ELISA were positive. The positive and negative serum samples for neosporosis (gifts from Prof. Lijun Jia of Yanbian University, Jilin, China) were set as a control to confirm the indirect ELISAs.

Statistical Analysis

To graph and analyze the data, the GraphPad Prism 8 software (GraphPad Software Inc., United States) was used. The prevalence and 95% confidence interval per pathogen species were calculated using the OpenEpi program (http://www.

TABLE 1 | The sampling sites of animals in the QTPA in this study.

State	Sampling site (altitude)	The number of collected and tested serum samples									
		Tibetan sheep	Yak	Cow	Pig	Cattle	Horse	Chicken	Camel	Donkey	Total
HB	Menyuan (2,866 m)	36	20	0	0	11	0	0	0	0	67
	Gangcha (3,827 m)	404	0	0	0	0	14	0	0	0	418
	Haiyan (3,000 m)	145	104	0	0	0	10	0	0	0	259
	Total	585	124	0	0	11	24	0	0	0	744
HN	Gonghe (3,200 m)	190	20	0	0	0	265	0	0	0	475
	Guide (2,200 m)	0	0	0	30	0	0	0	0	0	30
	Total	190	20	0	30	0	265	0	0	0	505
HX	Delingha (2,980 m)	0	0	0	30	45	0	0	0	0	75
	Golmud (2,780 m)	0	20	0	20	0	0	0	0	0	40
	Tianjun (3,993 m)	8	0	0	0	0	0	0	0	0	8
	Wulan (4,000 m)	0	0	0	0	0	0	0	49	0	49
	Total	8	20	0	50	45	0	0	49	0	172
YS	Zhiduo (4,897 m)	0	20	0	0	0	0	0	0	0	20
GL	Maqin (4,100 m)	0	110	0	0	50	0	0	0	0	160
	Darlag (4,271 m)	0	86	0	0	0	0	0	0	0	86
	Banma (3,970 m)	0	133	0	0	0	0	0	0	0	133
	Total	0	329	0	0	50	0	0	0	0	399
HUN	Jianzha (2,063 m)	45	0	0	0	45	0	0	0	0	90
	Henan (4,200 m)	0	0	0	0	0	60	0	0	0	60
	Total	45	0	0	0	45	60	0	0	0	150
HD	Huzhu (2,535 m)	21	40	0	48	0	0	30	0	37	176
	Ledu (2,000 m)	0	20	389	67	100	0	0	0	0	576
	Minhe (2,174 m)	0	0	107	0	0	0	30	0	0	137
	Pingan (2,183 m)	53	0	0	77	0	0	0	0	0	130
	Total	74	60	496	192	100	0	60	0	37	1,019
XN	Datong (2,756 m)	0	219	0	24	200	30	80	0	0	553
	Huangzhong (2,645 m)	0	0	0	68	0	10	40	0	0	118
	Huangyuan (2,660 m)	0	0	0	48	0	0	30	0	0	78
	Xining (2,261 m)	0	0	0	44	0	0	0	0	0	44
	Total	0	219	0	184	200	40	150	0	0	793
Total		902	792	496	456	451	389	210	49	37	3,782

HB, Haibei Tibetan Autonomous Prefecture, 36°44′-39°05′N and 98°5′-102°41′ E; HN, Hainan Tibetan Autonomous Prefecture, 34°38′-37°10′ N and 98°55–105°50′ E; HX, Haixi Mongolian and Tibetan Autonomous Prefecture, 35°01′-39°20′ N and 96°06′-99°42′ E; YS, Yushu Tibetan Autonomous Prefecture, 27°35′-36°35′ N and 89°35′-97°55′ E; GL, Guoluo Tibetan Autonomous Prefecture, 32°31′-35°37′ N and 96°54′-101°51′ E; HUN, Huangnan Tibetan Autonomous Prefecture, 34°04′-36°10′ N and 100°34′-102°28′ E; HD, Haidong city, 35°25.9′-37°05′ N and 100°41.5′-103°04′ E; XN, Xining city, 36°34′ N and 101°49′ E.

openepi.com/Proportion/Proportion.htm). A chi-squared test was conducted to compare the proportions of detected sample positivity in different regions and among different animals. Differences were considered to be statistically significant when resulting *P*-values were lower than 0.05.

RESULTS

Cut-Off Values

To develop the epidemiology of neosporosis in current animals from the Qinghai-Tibetan Plateau, the cut-off values of indirect ELISA methods based on the two antigens, r*Nc*SAG1 and r*Nc*GRA7, were calculated to analyze both *N. caninum*-specific IgG and IgM antibodies in this study (**Table 2**).

Indirect ELISAs

Here, the combination of r*Nc*SAG1 and r*Nc*GRA7 proteins was used to examine the serological prevalence of neosporosis in various animals in the Qinghai-Tibetan Plateau. The overall seroprevalence of *N. caninum* in the examined animals was 18.6 (705/3,782) and 48.9% (1,850/3,782) for the IgG and IgM antibodies, respectively (**Table 3** and **Figure 1**). Further analysis showed that out of the 3,782 animals, 330 (8.7%) were positive for both the IgG and IgM antibodies, and 2,275 (60.2%) were determined to be positive for at least one *N. caninum* indicator (**Table 3**). Moreover, the current study found that the 52% (469/902) of the Tibetan sheep, 46.7% (370/792) of the yaks, 67.3% (334/496) of the cows, 97.4% (444/456) of the pigs, 19.5% (88/451) of the cattle, 91.8% (357/389) of the horses, 61.9% (130/210) of the chickens, 85.7% (42/49) of the camels, and 97.3%

TABLE 2 | Cut-off values of current indirect ELISA tests in this study.

Animal	Antigen	Antibody	Cut-off value
Tibetan sheep	SAG1	IgG	0.373
		IgM	0.232
	GRA7	IgG	0.288
		IgM	0.254
Yak	SAG1	IgG	0.263
		IgM	0.137
	GRA7	IgG	0.234
		IgM	0.148
Cow	SAG1	IgG	0.273
		IgM	0.136
	GRA7	IgG	0.238
		IgM	0.149
Pig	SAG1	IgG	0.440
		IgM	0.120
	GRA7	IgG	0.376
		IgM	0.135
Cattle	SAG1	IgG	0.274
		IgM	0.149
	GRA7	IgG	0.248
		IgM	0.167
Horse	SAG1	IgG	0.262
		IgM	0.237
	GRA7	IgG	0.234
		IgM	0.401
Chicken	SAG1	IgG	0.397
		IgM	0.252
	GRA7	IgG	0.326
		IgM	0.145
Camel	SAG1	IgG	0.370
		IgM	–
	GRA7	IgG	0.288
		IgM	–
Donkey	SAG1	IgG	0.275
		IgM	–
	GRA7	IgG	0.268
		IgM	–

–, *not tested.*

(36/37) of the donkeys were positive for at least one parasitic indicator (IgG or IgM).

As shown in **Table 3** and **Figure 1**, to analyze the positive animals found that the donkey was the most prevalent animal (97.3%, 36/37) for IgG positivity, followed by pig (96.7%, 441/456), chicken (42.9%, 90/210), camel (14.3, 7/49), Tibetan sheep (12.4%, 112/902), cow (2.0%, 10/496), horse (0.8, 3/389), yak (0.5%, 4/792), and cattle (0, 0/451). While analysis for the IgM antibody positivity, the horse was the most prevalent animal (91.8%, 357/389) for IgM positivity, followed by cow (66.9%, 332/496), chicken (49.5%, 104/210), yak (46.8%, 371/792), Tibetan sheep (44.3%, 400/902), pig (36.8%, 168/456), and cattle (19.5, 88/451).

Analysis of Influence of Altitude on Seroprevalence of *N. caninum*

To analyze the influence of seroprevalence from different heights above sea levels in the sampling areas, all the animals were differentiated into three groups, namely the 2,000–3,000-, 3,000–4,000-, and 4,000–5,000-m altitude groups (**Table 4**). The analysis found significant differences ($P < 0.05$) from different altitudes in *N. caninum* specific-IgG in the Tibetan sheep, and IgM in the yak, but there was no difference in other current animals.

DISCUSSION

The SAG1 and GRA7 of *N. caninum* have been identified and tested as important candidates for serological diagnosis of neosporosis in animals (19–23). To develop the epidemiology of neosporosis in current animals from the Qinghai-Tibetan Plateau, indirect ELISA methods based on the two antigens were established to detect both *N. caninum*-specific IgG and IgM antibodies in this study. Although the SAG1 and GRA7 antigens of *N. caninum* were identified to be expressed in different stages of parasitic life cycles (19–23), a combination of recombinant protein-based ELISAs offers the best evidence for the diagnosis of *N. caninum* infection in this study. This study was the first to combine the SAG1 and GRA7 proteins in detection of *N. caninum*, and current ELISAs based on them confirmed that seropositive animals for neosporosis were present in the examined sampling areas. The results above showed that the animals in the QTPA present *N. caninum* infections, suggesting that the animals could have key roles in the transmission and prevalence of *N. caninum* in the plateau area. To the best of our knowledge, this is the first report of *N. caninum* infection in the present animals in the QTPA, the first epidemiology of neosporosis in the cow and camel in China, and the first record of *N. caninum* IgM antibodies in all the surveyed animals in China.

A variety of unique animals has been domesticated in the QTPA, which is the largest plateau with the highest average altitude on the planet, and the animals share water and food in the plateau area (24, 25). Infectious diseases caused by *N. caninum* parasites are common in animals in the world (1, 3, 5). Of the investigated animal species in this study, the IgG positivity was from 0 (cattle) to 97.3% (donkey), while that of IgM was from 19.5 (cattle) to 91.8% (horse). These findings showed the higher epidemiology of neosporosis in current animals in the QTPA compared with the 5.14, 8.4, 1.9, 23.1, and 9.5% IgG seroprevalence in yaks, Tibetan sheep, pigs, chickens, and equines in several provinces in China (12–18). Moreover, the *N. caninum* positivity rates of the present animals were also higher than the 13.46% in equines in the world (17), 40% in pigs in Italy (26), and 3.9% in camels in Iran (27). Interestingly, the current study found that 97.3% of the donkeys (36/37) and 96.7% of the pigs (441/456) were IgG-positive. Intermediate hosts probably become infected with *N. caninum* mainly through ingestion of foods or drinking water contaminated by sporulated *N. caninum* oocysts (1–3). Most of the pig samples in this study were collected from Xining and Haidong cities, and the sampling areas seem to present a large number of important risk factors

TABLE 3 | Seroprevalence of *Neospora caninum*- specific IgG and IgM in animals in the QTPA.

Animal	State	No.	Total IgG-positive (%, 95% CI)	Total IgM-positive (%, 95% CI)	Both IgG and IgM-positive (%, 95% CI)	Single-IgG-positive (%, 95% CI)	Single-IgM-positive (%, 95% CI)
Tibetan sheep	HB	585	103 (17.6, 14.5–20.7)	362 (61.9, 57.9–65.8)	80 (13.7, 10.9–16.5)	23 (3.9, 2.4–5.5)	282 (48.2, 44.2–52.3)
	HN	190	2 (1.1, 0.4–2.5)	7 (3.7, 1.0–6.4)	1 (0.5, 0.5–1.6)	1 (0.5, 0.5–1.6)	6 (3.2, 0.7–5.6)
	HX	8	0	0	0	0	0
	HUN	45	7 (15.6, 5.0–26.1)	31 (68.9, 55.4–82.4)	7 (15.6, 5.0–26.1)	0	24 (53.3, 38.8–67.9)
	HD	74	0	0	0	0	0
	Total	902	112 (12.4, 10.3–14.6)	400 (44.3, 41.1–47.6)	88 (9.8, 7.8–11.7)	24 (2.7, 1.6–3.7)	312 (34.6, 31.5–37.7)
Yak	HB	124	0	24 (19.4, 12.4–26.3)	0	0	24 (19.4, 12.4–26.3)
	HN	20	0	0	0	0	0
	YS	20	0	4 (20.0, 2.5–37.5)	0	0	4 (20.0, 2.5–37.5)
	HX	20	0	0	0	0	0
	GL	329	2 (0.6, 0.2–1.4)	197 (59.9, 54.6–65.2)	2 (0.6, 0.2–1.4)	0	195 (59.3, 54.0–64.6)
	HD	60	2 (3.3, 1.2–7.9)	31 (51.7, 39.0–64.3)	1 (1.7, 1.6–4.9)	1 (1.7, 1.6–4.9)	30 (50.0, 37.3–62.7)
	XN	219	0	115 (52.5, 45.9–59.1)	0	0	115 (52.5, 45.9–59.1)
	Total	792	4 (0.5, 0.0–1.0)	371 (46.8, 43.4–50.3)	3 (0.4, 0.0–0.8)	1 (0.1, 0.1–0.4)	368 (46.5, 43.0–49.9)
Cow	HD	496	10 (2.0, 0.8–3.3)	332 (66.9, 628–71.1)	7 (1.4, 0.4–2.4)	3 (0.6, 0.1–1.3)	325 (65.5, 61.3–69.7)
	Total	496	10 (2.0, 0.8–3.3)	332 (66.9, 628–71.1)	7 (1.4, 0.4–2.4)	3 (0.6, 0.1–1.3)	325 (65.5, 61.3–69.7)
Pig	HN	30	25 (83.3, 70.0–96.7)	6 (20.0, 5.7–34.3)	6 (20.0, 5.7–34.3)	19 (63.3, 46.1–80.6)	0
	HX	50	50 (100, 100–100.0)	25 (50.0, 36.1–63.9)	25 (50.0, 36.1–63.9)	25 (50.0, 36.1–63.9)	0
	HD	192	190 (99.0, 97.5–100.4)	62 (32.3, 25.7–38.9)	62 (32.3, 25.7–38.9)	128 (66.7, 60.0–73.3)	0
	XN	184	176 (95.7, 92.7–98.6)	75 (40.8, 33.7–47.9)	73 (39.7, 32.6–46.7)	103 (56.0, 48.8–63.2)	2 (1.1, 0.4–2.6)
	Total	456	441 (96.7, 95.1–98.3)	168 (36.8, 42.4–41.3)	166 (36.4, 32.0–40.8)	275 (60.3, 55.8–64.8)	2 (0.4, 0.2–1.0)
Cattle	HB	11	0	3 (27.3, 1.0–53.6)	0	0	3 (27.3, 1.0–53.6)
	HX	45	0	4 (8.9, 0.6–17.2)	0	0	4 (8.9, 0.6–17.2)
	HUN	45	0	23(51.1, 36.5–65.7)	0	0	23(51.1, 36.5–65.7)
	GL	50	0	10 (20.0, 8.9–31.1)	0	0	10 (20.0, 8.9–31.1)
	HD	100	0	25 (25.0, 16.5–33.5)	0	0	25 (25.0, 16.5–33.5)
	XN	200	0	23(11.5, 7.1–15.9)	0	0	23(11.5, 7.1–15.9)
	Total	451	0	88 (19.5, 15.9–23.2)	0	0	88 (19.5, 15.9–23.2)
Horse	HB	24	0	24 (100, 100.0–100.0)	0	0	24 (100, 100.0–100.0)
	HN	265	1 (0.4, 0.4–1.1)	235 (88.7, 84.9–92.5)	1 (0.4, 0.4–1.1)	0	234 (88.3, 84.4–92.2)
	HUN	60	2 (3.3, 1.2–7.9)	59 (98.3, 95.1–101.6)	2 (3.3, 1.2–7.9)	0	57 (95.0, 89.5–100.5)
	XN	40	0	39 (97.5, 92.7–102.3)	0	0	39 (97.5, 92.7–102.3)
	Total	389	3 (0.8, 0.1–1.6)	357 (91.8, 89.0–94.5)	3 (0.8, 0.1–1.6)	0	354 (91.0, 88.2–93.8)
Chicken	HD	60	36 (60.0, 47.6–72.4)	31 (51.7, 39.0–64.3)	29 (48.3, 35.7–61.0)	7 (11.7, 3.5–19.8)	2 (33.0, 1.2–7.9)
	XN	150	54 (36.0, 28.3–43.7)	73 (48.7, 40.7–56.7)	34 (22.7, 16.0–29.4)	20 (13.3, 7.9–18.8)	39 (26.0, 19.0–33.0)
	Total	210	90 (42.9, 36.2–49.6)	104 (49.5, 42.8–56.3)	63 (30.0, 23.8–36.2)	27 (12.9, 8.3–17.4)	41 (19.5, 14.2–24.9)
Camel	HX	49	7 (14.3, 4.5–24.1)	–	–	7 (14.3, 4.5–24.1)	–
	Total	49	7 (14.3, 4.5–24.1)	–	–	7 (14.3, 4.5–24.1)	–
Donkey	HD	37	36 (97.3, 92.1–102.5)	–	–	36 (97.3, 92.1–102.5)	–
	Total	37	36 (97.3, 92.1–102.5)	–	–	36 (97.3, 92.1–102.5)	–
Total		3,782	703 (18.6, 17.3–19.8)	1,820 (48.1, 46.5–49.7)	330 (8.7, 7.8–9.6)	373 (9.9, 8.9–10.8)	1,490 (39.4, 37.8–41.0)

No., No. of tested in this study; %, prevalence; 95% CI, 95% confidence interval; HB, Haibei Tibetan Autonomous Prefecture; HN, Hainan Tibetan Autonomous Prefecture; HX, Haixi Mongolian and Tibetan Autonomous Prefecture; YS, Yushu Tibetan Autonomous Prefecture; GL, Guoluo Tibetan Autonomous Prefecture; HUN, Huangnan Tibetan Autonomous Prefecture; HD, Haidong city; XN, Xining city.

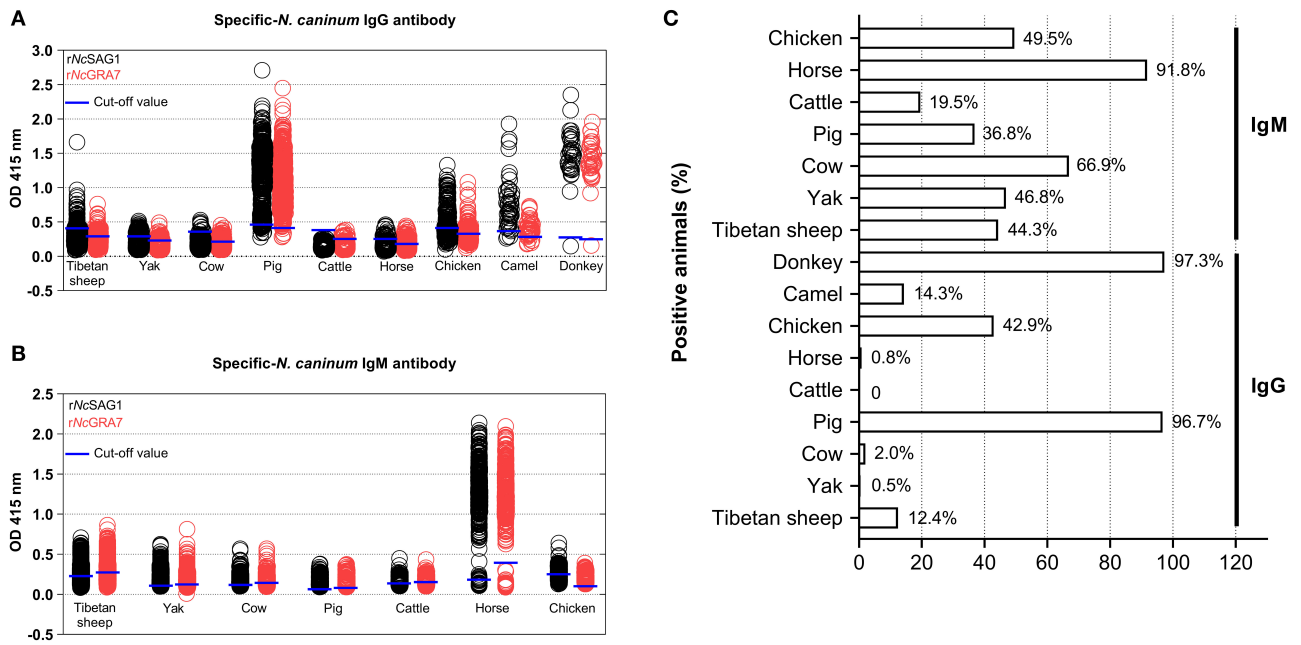

FIGURE 1 | *Neospora caninum*-specific IgG and IgM antibodies in various animals in the Qinghai-Tibet Plateau Area were detected by indirect ELISA methods based on the *Nc*SAG1 and *Nc*GRA7 antigens in this study. The blue short lines represented the cut-off values. **(A)** *N. caninum*-specific-IgG antibodies, **(B)** *N. caninum*-specific IgM antibodies, and **(C)** positive animals for neosporosis (%).

such as stray dogs for *N. caninum* infection (28). Moreover, the current pig farms in the Qinghai-Tibet Plateau are non-intensive farming, which means more convenient conditions for *N. caninum* oocysts seeded by the dogs are exposed to pigs. For donkeys, they were likely exposed to foods and water contaminated with oocysts in Haidong city, while the limitation of this study is that the sample size is too small for donkeys. On the other hand, although *N. caninum* is a major pathogen of abortion and reproduction problems in cattle and dogs (1–3), its infection in various animal species including the current animals has been reported (7–18). However, this study found higher IgM positivity rates in the horses (91.8%) and chickens (49.5%) than in the yaks and cattle. Actually, the DNAs of *N. caninum* have been molecularly amplified in horses and chickens (12, 17, 29), and it is possible that the current detection of high IgM positivity rate may indicate the possibility that neosporosis is developing, but this must be combined with clinical observations and isolation of *N. caninum* parasites to confirm. The present serological results indicate that the Qinghai-Tibet Plateau is also an endemic area of *N. caninum*, which may be related to the existence of a large number of stray dogs and herding sheepdogs there.

Generally considered the IgG antibodies rise to protective levels after infection and remain detectable for years while the lower occurrence of IgM antibodies is within days to a couple of weeks, moreover, *N. caninum* positive for IgG + IgM are proposed to be chronic reactivated cases. The current study found that the seroprevalence of the *N. caninum* IgM antibody in Tibetan sheep, yaks, cows, horses, cattle, and chickens was higher than that of the IgG antibody, but in pigs IgG was lower. Moreover, 36.4% (166/456) of the pigs and 30% (63/210) of the chickens were tested to be both IgG- and IgM-positive. Furthermore, the current results found low IgG positivity rates present in the cattle (0), yaks (0.5%) and cows (2.0%) but more than 19.5% IgM positivity rates in the animals. Therefore, the present study may suggest the prevalence of acute neosporosis in Tibetan sheep, yaks, cows and horses, chronic re-emergence of neosporosis in pigs and chickens, and chronic neosporosis in cattle in the testing area. These reveal that the animal populations are suffering from *N. caninum* infection or have become carriers of *N. caninum* antibodies after the infection.

To show the influence on seroprevalence from different heights above sea levels of the sampling areas in the 2,000–3,000, 3,000–4,000, and 4,000–5,000 m altitudes in this study, an analysis was conducted. It was found that there were only significant differences ($P < 0.05$) from different altitudes in *N. caninum*-specific-IgG in Tibetan sheep and IgM in yak, but that there was no difference in other current animals. Nonetheless, because of the limitation of sampling sites and the number of samples, it cannot be said that altitude is a key factor affecting the prevalence of *N. caninum* in a strict sense. But the differences present may be because of the different feeding methods for various animals: the animals are grazing in the high-altitude areas sharing the common waters and foods, while a large number of humans and the definitive-host dogs especially stray dogs are activating in the low altitude areas leading to these food-borne

TABLE 4 | Analysis of the influence of altitude on the seroprevalence of the *Neospora caninum* IgG and IgM antibodies and distribution of toxoplasmosis and neosporosis in the QTPA.

Animal	Antibody	2,000–3,000 m		3,000–4,000 m		4,000–5,000 m		*P*-value
		Tested	Positive (%)	Tested	Positive (%)	Tested	Positive (%)	
Tibetan sheep	IgG	155	11 (7.1)	747	103 (13.8)	0	–	0.0403
	IgM		67 (43.2)		363 (48.6)		–	0.4631
Yak	IgG	319	2 (0.6)	257	0	216	2 (0.9)	0.3427
	IgM		166 (52.0)		50 (19.5)		155 (71.8)	<0.0001
Cow	IgG	496	10 (2.0)	0	–	0	–	–
	IgM		332 (66.9)		–		–	–
Pig	IgG	456	441 (96.7)	0	–	0	–	–
	IgM		168 (36.8)		–		–	–
Cattle	IgG	401	0	0	–	50	0	–
	IgM		78 (19.5)		–		10 (20.0)	0.9397
Horse	IgG	40	0	289	1 (0.3)	60	2 (3.3)	0.0517
	IgM		39 (97.5)		259 (89.6)		59 (98.3)	0.8645
Chicken	IgG	210	90 (42.9)	0	–	0	–	–
	IgM		104 (49.5)		–		–	–
Camel	IgG	0	–	0	–	49	7 (14.3)	–
Donkey	IgG	37	36 (97.3)	0	–	0	–	–

–, not tested; %, prevalence; n, no significant difference.

animals have frequently exposed the infecting source and cause *N. caninum* infections.

In conclusion, this study is the first to demonstrate *N. caninum* infections using serological ELISAs based on the combination of recombinant SAG1 and GRA7 proteins in various animals in the current plateau area, and determination of the *N. caninum* IgM antibody in these animals in China. These give latest valuable data on the epidemiology of neosporosis in China and in plateau areas of the world. Future studies should focus on clinical cases of neosporosis such as abortions and neurological disorders in animals and perform isolation of *N. caninum* parasites in domestic and wild animals including definitive and intermediate hosts in the Qinghai-Tibetan Plateau Area.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Qinghai University (No. SL-2021016, date of approval: March 16, 2021). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

TQ: conceptualization, data curation, formal analysis, investigation, writing (original draft), and writing (review and editing). JA and JY: data curation, formal analysis, and investigation. HZ: investigation, formal analysis, and validation. YYZ, YLZ, HMZ, and QQ: data curation and formal analysis. MK and YS: resources and writing (review and editing). JL: conceptualization, funding acquisition, resources, writing (original draft), and writing (review and editing). All authors contributed to the article and approved the submitted version.

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Acaricidal Properties of Four Neem Seed Extracts (*Azadirachta indica*) on the Camel Tick *Hyalomma dromedarii* (*Acari: Ixodidae*)

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Tick infestation remains one of the major health problems that affect the productivity and comfort of camels. The control of ticks mainly relies on using chemical acaracides. Limited information is available on the potential benefits and activity of various neem extracts on *Hyalomma* ticks. The present study investigated the acaricidal activity of neem seed extracts at different concentrations against developmental stages of the camel tick *Hyalomma dromedarii* in comparison to Butox and diazinon. The acaricidal activity of three extracts, namely, hexane extract (HE), methyl chloride extract (MCE), and methanol extract (ME), of neem seeds (*Azadirachta indica*) were tested at varying concentrations of 5, 10, 15, and 20% on engorged *H. dromedarii* female ticks at days 1, 3, 5, 7, 12, 16, 20, 28, 37, and 43 after treatment (DPT). Interestingly, results of applying different neem seed extracts to engorged *H. dromedarii* female ticks showed that the most effective extract was hexane at concentration 20%, causing 100% mortality at 1st day post-application, while methanol extract at 20% and dichloromethane extract at 20% caused the death of all ticks at 28th day posttreatment as compared to Butox® 5.0 and Diazinon-60, which resulted in mortality of all ticks at 3 and 5 DPT, respectively. In addition, no mortality was reported with the application of aqueous extract (AE), which served as the control group. Furthermore, the neem hexane extract exhibited high efficacy against reproductive performance of female ticks, whereas no fertility or oviposition was reported at all of their concentrations. Additionally, no hatchability occurred using all neem extracts, except the aqueous extract, which showing no effect. In the present study, larvae responded more rapidly to the plant extracts, whereas mortality

of all larvae was recorded at 24 h after treatment with 5% hexane. Taken together, this study pointed out that the acaricidal effect of hexane extract of neem seeds was more effective and could be economically used for controlling *H. dromedarii* ticks.

Keywords: acaricidal properties, neem seed extracts, eggs, immature, adult, camel tick

INTRODUCTION

Camels are considered elemental part in the sophistication and farming of many countries around the world, being a good source of milk and meat, and serve as a means of transportation (1, 2). However, their productivity is hindered by a wide range of external and internal parasites, resulting in considerable economic losses (1, 3–6). Ticks are destructive blood-sucking ectoparasites of worldwide distribution and of a greater economic importance in tropical and subtropical areas (7, 8). Ticks have been considered potent vectors for the transmission of various bacterial, viral, and parasitic diseases of veterinary and medical importance. Therefore, ticks are considered a major contributor for several emerging and re-emerging diseases (9, 10). Given its important economic impacts, tick infestation in camels might result in a series of symptoms, ranging from mild to severe anemia; intense pruritus and deterioration the skin of affected animals; loss of appetite, leading to a reduction in growth rate and decreased productivity; and occasional mortalities in untreated and young animals, and therefore, they result in considerable economic losses (11, 12). To the authors' knowledge, *Hyalomma dromedarii* is considered the most reported *Hyalomm*a spp. parasitizing camels in Egypt (13). The camel is considered the primary host containing the adult stage of *Hyalomm*a spp.; however, it also infests other domestic animals, including cattle, sheep, goats, and equids. The remaining stages, including larvae and nymphs, feed on birds and small burrowing animals, but nymphs can infest large animals such as adults and complete its life cycle on two or three hosts (14).

The control of ticks is mainly based on the direct application or injecting of acaricides to animals. Several acaricides have been extensively used and recommended for the control of ticks including organophosphates, carbamates, pyrethroids, and amidines (15). However, several major drawbacks were reported for the majority of acaricides including alarming reports of tick resistance, residues in foods, and environmental pollution (16, 17). It is therefore not surprising to mention that there is an urgent need to develop eco-friendly effective alternatives for these chemicals. Interestingly, the use of herbal medications becomes a promising alternative approach for the treatment of various infectious agents because of their biodegradability, target efficiency, and cost-effectiveness, and therefore, they gained a considerable interest in tropical and subtropical regions (18–26). Among others, plant-derived materials and their bioactive substances were proposed as substitutes for synthetic acaricides due to their activity against ticks (27). As compared to synthetic ones, several previous reports revealed that herbal acaricides caused little environmental pollution and a low toxicity level to non-target organisms including humans, apart from the rapid biodegradation of their residues and their role in prevention of resistance development (28, 29).

Neem is considered one of the most reliable botanical sources of biopesticides with a wide range of biological activities (30). The neem tree, *Azadirachta indica A. Juss*, is an evergreen tree that originates from India and other neighboring countries (31–33). The functional ingredients of neem, including neem oil, bark, leaves, and their purified biochemical products, exhibited promising therapeutic effects and showed anticancer, anti-inflammatory, and antimicrobial properties (32–35). It is noteworthy to state that neem and its extracts showed potent activity against all the stages (adult, nymph, and larva) of ticks (29, 36, 37). Azadirachtin analogs, including azadirachtin A and its B counterpart, are among the most potent constituents isolated from the crude extracts of neem (38, 39). These analogs exhibited a wide range of biological properties, which include antifeedant, larvicidal, ovicidal, repellent action, growth deregulation, reduction in ecdysone levels, alterations in development and reproduction, sterility, and damage in molting processes (40). In accordance with its mechanisms of action against insects, neem is structurally analogous to insect hormones known as "ecdysone," which are responsible for metamorphosis in insects. It is therefore not surprising to mention that the larvicidal and acaricidal properties of neem are the underlying bases for the use of neem products for control of agricultural pests.

Revising the available literature, limited information is available about the use of *A. indica* (neem) extracts against *H. dromedarii*. A previous study (41) showed the *in vitro* acaricidal activity of the methanolic extract of neem leaves against engorged adult female ticks, egg hatchability, and larvae of camel ticks (*H. dromedarii*) using the immersion method and in mortality rates of engorged ticks from 1st to 15th DPT (up to 100%), with some changes in morphology. Also, there was a potent activity of the methanolic extract of neem leaves on hatchability of *H. dromedarii* eggs (100%) from 1st to 15th DPT, and it induced 100% mortality on the newly hatched larvae of *H. dromedarii* ticks. Agreed with the aforementioned information, this study aimed to investigate the effect of neem extract application on ticks infesting one-humped camels in Aswan Governorate, Egypt, which might provide new insights into the control of hard ticks in camels.

MATERIALS AND METHODS

Ethical Considerations

The ethical approval of the present study was obtained from the Research, Publication, and Ethics Committee of the Faculty

of Veterinary Medicine, Aswan University, Egypt, and the Institutional Review Approval Board Number is 2020/5.

Collection of Hard Ticks

Engorged adult, larvae, and nymphs of hard ticks were collected from naturally infested camels (5- to 15-year-olds) from Daraw market, Daraw city, Aswan Governorate, Egypt. In order to minimize damage to the mouthparts and cuticle, the ticks were manipulated by rotating for easy removal with a pair of soft forceps. The collected ticks were then placed in a clean plastic container with perforated lids to allow ventilation, then immediately transported to the laboratory at the Department of Parasitology, Faculty of Veterinary Medicine, Aswan University, for identification using standardized international keys and bioassays, as described elsewhere (14, 42, 43).

Chemical Materials

Hexane, diethyl ether, dichloromethane, and methanol solvents, Tween 20, Tween 80, TLC plates 20 × 20 cm, and vanillin spray reagent were purchased from Sigma Chemical Company, Cairo, Egypt. Synthetic chemical acaricides such as deltamethrin (Butox 5%) and Diazinon-60 were purchased from Arab Chemical Industrial Company, Cairo, Egypt.

Plant Preparation and Extraction

Collection of Seeds

Neem seeds were collected from an old neem tree located at Aswan University during July 2020 at 10 a.m. and identified and authenticated by Department of Botany, College of Science, Aswan University. The collected seeds were then transported to the Laboratory of Parasitology, Faculty of Veterinary Medicine, Aswan University, Egypt, and stored at 4°C until analysis.

Preparation of Seeds

The seeds were cleaned to remove any sticks, unwanted leaves, bad seeds, sand and dirt in order to ensure the oil produced is not contaminated and of high quality. The cleaned neem seeds were dried at 55°C for 72 h until constant weight and moisture content determined, as described elsewhere (44) using Equation 1:

$$\text{Moisture content} = \frac{\text{W1 W2}}{\text{W1}} \times 100\%$$

where W1 is the weight of neem seeds before drying and W2 is the weight of neem seeds after drying.

The dried clean neem seeds were roasted about 5 min to enhance extraction of oil, then crushed in a blender, and sieved to obtain particles ranging from 425 to 710 μm in size. The sieved neem powder was then stored under vacuum in an airtight container at 4°C prior to use (45).

Neem Seed Extraction by Cold Maceration

To obtain extracts from the neem seeds, about 9,000 ml of hexane was added to 3,000 g of the dried grounded neem seeds in a conical flask, which is then allowed to stand at room temperature for a period of 3 days with intermittent agitation (stirring) until the soluble matter dissolved. This mixture was filtered by gravity filtration, producing a hexane extract mixture as the filtrate, which was then concentrated using a rotary evaporator and stored at 4°C until further use (46). For the study, three more consecutive extracts, including dichloromethane, methanol, and aqueous extracts from residues have been collected using the same method. The yielded neem oil was calculated elsewhere (47) using the following equation (Equation 2).

$$\text{Extraction yield (\%)} = \frac{\text{M1} - \text{M2}}{\text{M1}} \times 100\%$$

where M1 is the mass of the neem seed before extraction and M2 is the mass of the neem seed after extraction.

Preparation of Emulsions

The emulsion was prepared by mixing neem oil and two different non-ionic surfactants (Tween 20 and Tween 80) using each emulsifier separately, at rates of 1:5 and 1:3, respectively. Surfactants and deionized water were first mixed using a stirrer, and then neem oil was added. This step was followed by preparation of the different concentrations (5, 10, 15, and 20%) from each extracts as described elsewhere (48) with slight modification in which Tween 20 and Tween 80 were replaced by soap as a surfactant for complete blending of neem oil with water.

Preparation of Thin-Layer Chromatography (TLC) Plates

In this step, the plant extract was spotted on the plate with the aid of a capillary tube. The spot was applied 1 cm upward from the lower end of the TLC plate, then placed in a beaker consisting hexane:ether [(1:1), (2:1), (3:1), (5:1), and (6:1)] with drops of methanol and dichloromethane:methanol (7:1/4) as the mobile phase. Run spots were performed to separate the compounds. Later on, when the mobile phase reached the upper end of the TLC plate, it was removed from a beaker and was air dried. The TLC run plates were observed by using a UV spectrophotometer, and the separated spots were marked. Then, the spots were visualized by vanillin spray reagent. After spraying, the plates were immediately placed in the oven maintained at 1,100°C for 5–10 min (49). A preparative thin-layer chromatographic separation of the dichloromethane extract was applied.

In vitro Evaluation the Acaricidal Effect of Neem on Hard Ticks of Camels

In vitro Evaluation of Acaricidal Effect of Neem Seed Extracts on Adult Female *H. dromedarii*

Four concentrations (5, 10, 15, and 20%) of neem extract emulsions were used, while in case of aqueous emulsion, only two concentrations of 5 and 10% were tested. Also, 5% deltamethrin (Butox 5%) and Diazinon-60 were applied. The tested groups included 15 adult female ticks (three replicates for each concentration) that were weighed and immersed in their respective dilutions for 5 min. The adult immersion test was then performed by placing each group of ticks in separate petri dishes, and all plates were incubated at 27–30°C and 70%−80% relative humidity (RH). A negative control composed of the surfactant and distilled deionized water was included along the study. The number of live and dead ticks was counted during the period of 43 days (posttreatment period). The mortality rate of adult

female ticks was calculated according to the following equation (Equation 3):

$$\text{Mortality rate} = \frac{\text{Number of dead ticks}}{\text{Total number of ticks}} \times 100$$

The acaricidal efficacy was calculated using the following equation (50), and the index of egg laying was calculated after completed oviposition (36 days), using the following formula (Equation 4), as described elsewhere (51):

$$\text{Index of egg laying (IE)} = \frac{\text{Mean weight of eggs laid (g)}}{\text{Weight of females (g)}}$$

The eggs were weighted and then incubated in test tubes at 27–30 °C and 70–80% RH. Later on, the percentage inhibition of egg laying was calculated after hatching 21 days by using the following formula (Equation 5):

$$\text{Inhibition of egg laying (\%)} = \frac{\text{IE control group} - \text{IE treated group}}{\text{IE control group}} \times 100.$$

Evaluation the Acaricidal Effect of Neem Seed Extracts on the Eggs of *H. dromedarii*

The neem extract emulsions with the different concentrations (5, 10, 15, and 20%) were applied to its corresponding group of eggs, which were laid by a control group. The eggs (5-day-old eggs) were immersed in neem seed extract emulsions for 5 min and then were placed in open test tubes until drying emulsions, and then the test tubes were closed with cotton plugs to avoid contamination. All tubes were also incubated at 27–30°C and RH of 70%−80%. The control group eggs consisted of filter paper envelops immersed in the surfactant, and distilled deionized water was also used. After 21 days, all tubes were incubated at 40°C for counting, and hatchability was determined, as described elsewhere (52).

Evaluation of Acaricidal Effect of Neem Seed Extracts on the Larva of *H. dromedarii*

The eggs of the adult control group were placed in test tubes and incubated at 27–30°C and 70%−80% RH until they hatched into larvae. The larval packet test was performed using about 300–400 larvae (at 10-day-old larvae) which were then placed in filter paper envelopes immersed in neem seed extract emulsions with the different concentrations (5, 10, 15, and 20%) (53). All envelopes were kept under the same incubating conditions. Reading of the results was recorded under UV light with a magnifying glass, and larvae with no movement were considered dead. Furthermore, the mortality rate was calculated as described elsewhere (54) according to the following equation (Equation 6):

$$\text{Mortality rate} = \frac{\text{Number of dead ticks}}{\text{Total number of ticks}} \times 100$$

Evaluation of Acaricidal Effect of Neem Seed Extracts on Nymphs of *H. dromedarii*

This step involved collection of nymphs from camel tail and ears. Each group of 15 nymphs was immersed in the various concentrations (5, 10, 15, and 20%) of neem extract emulsions for 5 min. Then, the nymphs were placed in separate petri dishes, and all plates were incubated at 27–30°C and 70%−80% RH. A negative control consisted of the surfactant, and distilled deionized water was also included. The number of live and dead nymphs of ticks was counted posttreatment during the whole time of the test, and the acaricidal efficacy was determined (50), using the following equation (Equation 7), and this step was performed on three replicates for each concentration.

$$\text{Mortality rate} = \frac{\text{Number of dead ticks}}{\text{Total number of ticks}} \times 100.$$

Statistical Analysis

The data related to effects of treatment using different concentrations of various neem seed extracts against the different stages of ticks (eggs, larva, nymph, and adult) and non-hatching eggs and antifeedant activity of the ticks were analyzed by one-way ANOVA. This was followed by using of Duncan multiple range tests (55) to determine the significant differences between treatments, and the least significant difference (LSD) test was used to separate the mean values, which were significant at the 95% level with Statistical Package for the Social Sciences (SPSS version 25). The level of significant association between treatments was considered at $p < 0.05$. Curves and the lethal concentrations (LC50 and LC90) were obtained at 95% using probit analysis with LDP line software (56).

RESULTS

Acaricidal Activity of Neem Seeds at Different Concentrations Against Engorged *Hyalomma dromedarii*

Data presented in **Table 1** show the effect of neem seed extracts, hexane extract (HE), dichloromethane extract (DE), methanol extract (ME), and aqueous extract (AE), used at a concentration of 5% as compared to the effect of Butox 5% and Diazinon-60 against *H. dromedarii* infestation in camels. As shown, the 5% hexane extract was the most effective extract, which showed evident changes and caused death of *Hyalomma* ticks from the first day of application with a percentage of 46.7%, and caused complete mortality of ticks at day 20. The methanol extract (ME) followed HE in its efficacy since and caused 100% mortality at day 37. On the other hand, little effect was obtained by uding dichloromethane, compared to that recorded with Butox® 5.0 and Diazinon-60, which caused mortality of all ticks at day 3 and day 5, respectively. By contrast, no mortality was recorded among control groups and following treatment with aqueous extracts. The data presented in **Table 2** show that hexane extract 10% was the most effective extract, causing mortality to 90% of hard ticks on the first day of application and the death of all ticks at day 5, followed by methanol extract which resulted in 100% mortality at day 28, while little effect was obtained

TABLE 1 | *In vitro* mortality rates of engorged *Hyalomma dromedarii* treated with 5% hexane, methanol, dichloromethane, and aqueous extracts of neem seeds in comparison to some synthetic chemical materials (Butox 5%, Diazinon-60).

Group	Days post treatment									
	1	3	5	7	12	16	20	28	37	43
HE	46.7^{ef}	53.3^{def}	60^{de}	66.7^{cd}	86.7^{ab}	93.3^{ab}	100^{a}	–	–	–
DE	0^{h}	0^{h}	6.7^{gh}	13.3^{gh}	20^{g}	20^{g}	20^{g}	20^{g}	66.7^{cd}	100^{a}
ME	0^{h}	0^{h}	13.3^{gh}	20^{g}	40^{f}	40^{f}	40^{f}	80^{bc}	100^{a}	–
AE	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	80^{bc}	100^{a}
C	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	66.7^{cd}	100^{a}
B	86.7^{ab}	100^{a}	–	–	–	–	–	–	–	–
D	53.3^{def}	80^{bc}	100^{a}	–	–	–	–	–	–	–

Small superscript letters (a, b, c, d, e, f, g, and h) in the columns and the rows denote significant difference ($P < 0.05$).
DPT, days posttreatment; DE, dichloromethane neem seed extract emulsion; HE, hexane neem seed extract emulsion; AE, aqueous neem seed extract emulsion; ME, methanol neem seed extract emulsion; C, control; D, Diazinon; B, Butox.

TABLE 2 | *In vitro* mortality rates of engorged *Hyalomma dromedarii* treated with 10% hexane, methanol, and dichloromethane extracts of neem seeds.

Group	Days post treatment									
	1	3	5	7	12	16	20	28	37	43
HE	90^{a}	90^{a}	100^{a}	–	–	–	–	–	–	–
DE	0^{f}	0^{f}	10^{e}	10^{e}	10^{e}	10^{e}	10^{e}	10^{e}	100^{a}	–
ME	40^{d}	40^{d}	50^{c}	80^{b}	90^{a}	90^{a}	90^{a}	100^{a}	–	–
AE	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	80^{bc}	100^{a}
C	0^{f}	0^{f}	0^{f}	0^{f}	0^{f}	0^{f}	0^{f}	0^{f}	66.7^{c}	100^{a}

Small superscript letters (a, b, c, d, e, and f) in the columns and the rows denote significant difference ($P < 0.05$).
HE, hexane neem seed extract emulsion; DE, dichloromethane neem seed extract emulsion; ME, methanol neem seed extract emulsion; C, control; AE, aqueous neem seed extract emulsion.

by dichloromethane extract. On the other hand, no mortality was recorded among control groups and because of treatment with aqueous extracts. Data presented in **Table 3** indicate that 15% concentration of hexane extract of neem seeds revealed significantly higher mortality (90%) of ticks at the first day of application and 100% mortality at the third day, followed by 15% methanol extract which caused 100% mortality at day 28, while little effect was obtained by 15% dichloromethane extract. As shown in **Table 4**, 20% concentration of hexane extract of neem seeds was the most toxic extract and triggered 100% mortality of ticks at the first day of application, while the same concentration of methanol and dichloromethane extracts evoke a similar effect but at day 28.

Effects of Various Concentrations of Neem Seed Extracts on Oviposition, Fertility, and Hatchability

Table 5 shows the reproductive performance, including oviposition and fertility percentage, of *H. dromedarii* engorged female ticks exposed to the effect of various concentrations of neem seed extracts. The results indicated that there was no oviposition with all concentrations of hexane extract, while oviposition percentages with application of methanol extract at concentrations of 5, 10, 15, and 20% were 18.8, 12.5, 7.5, and 2.5%, respectively. Furthermore, a little effect was obtained with the application of dichloromethane as the oviposition percentages were 87.5, 81.3, 75, and 68.8% at concentrations of 5, 10, 15, and 20%. The results also indicated that there was a significant effect for hexane extract on the fertility of *H. dromedarii* at all concentrations. In this regard, the fertility rates were 100, 95, 91.6, and 75%, and their corresponding concentrations of methanol extract concentrations were 5, 10, 15, and 20%, respectively. On the other hand, no effect was obtained upon application of aqueous extracts on oviposition and fertility of ticks. Data presented in **Table 5** also show that the hatching rates reached 0% with the application of all extract concentrations, except aqueous extract that has no inhibitory effect.

Effect of Various Concentrations of Neem Seed Extracts on *Hyalomma dromedarii* Larvae and Nymphs

Data presented in **Table 6** show that the hexane extract of neem seeds, at all concentrations, induced high mortality rates of newly hatched larvae at the first day posttreatment, followed by methanol extract which caused death of all hatched larvae using concentrations of 10 and 15% first day posttreatment, and the mortality rate was 80% at 1st DPT and reached 100% at the third day of application of 5% methanol extract, while the lowest effect was attained by dichloromethane extract

TABLE 3 | *In vitro* mortality rates of engorged *Hyalomma dromedarii* treated with 15% hexane, methanol, and dichloromethane extracts of neem seeds.

Group	Days post treatment									
	1	3	5	7	12	16	20	28	37	43
HE	90^{a}	100^{a}	–	–	–	–	–	–	–	–
DE	0^{f}	10^{e}	10^{e}	10^{e}	10^{e}	10^{e}	10^{e}	10^{e}	100^{a}	–
ME	40^{d}	40^{d}	50^{c}	80^{b}	90^{a}	90^{a}	90^{a}	100^{a}	–	–
AE	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	80^{bc}	100^{a}
C	0^{f}	0^{f}	0^{f}	0^{f}	0^{f}	0^{f}	0^{f}	0^{f}	66.7^{c}	100^{a}

Small superscript letters (a, b, c, d, e, and f) in the columns and the rows denote significant difference ($P < 0.05$).
HE, hexane neem seed extract emulsion; DE, dichloromethane neem seed extract emulsion; ME, methanol neem seed extract emulsion; C, control; AE, aqueous neem seed extract emulsion.

TABLE 4 | *In vitro* mortality rates of engorged *Hyalomma dromedarii* treated with 20% hexane, methanol, and dichloromethane extracts of neem seeds.

Group	Days post treatment									
	1	3	5	7	12	16	20	28	37	43
HE	100^{a}	–	–	–	–	–	–	–	–	–
DE	0^{d}	20^{c}	20^{c}	20^{c}	20^{c}	20^{c}	20^{c}	100^{a}	–	–
ME	70^{b}	80^{ab}	90^{a}	90^{a}	90^{a}	90^{a}	90^{a}	100^{a}	–	–
AE	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	0^{h}	80^{bc}	100^{a}
C	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}	66.7^{b}	100^{a}

Small superscript letters (a, b, c, d, e, and f) in the columns and the rows denote significant difference ($P < 0.05$).
HE, hexane neem seed extract emulsion; DE, dichloromethane neem seed extract emulsion; ME, methanol neem seed extract emulsion; C, control; AE, aqueous neem seed extract emulsion.

TABLE 5 | Effect of 5, 10, 15, and 20% neem seed extracts on reproductive performance of *Hyalomma dromedarii* including oviposition, fertility, and hatchability.

Criteria		Extracts and their concentration															
		5%				10%				15%				20%			
		HE	DE	ME	AE	HE	DE	ME	AE	HE	DE	ME	AE	HE	DE	ME	AE
Oviposition		0^{a}	87.5^{h}	18.8^{d}	100^{i}	0^{a}	81.3^{g}	12.5^{c}	100^{i}	0^{a}	75^{f}	7.5^{b}	100^{i}	0^{a}	68.8^{e}	2.5^{a}	100^{i}
Fertility		0^{a}	100^{e}	100^{e}	100^{e}	0^{a}	91.5^{d}	95^{de}	100^{e}	0^{a}	75^{c}	91.6^{d}	100^{e}	0^{a}	68.2^{b}	75^{c}	100^{e}
Hatchability	Days	HE	DE	ME	AE	HE	DE	ME	AE	HE	DE	ME	AE	HE	DE	ME	AE
	15	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	21	0^{a}	0^{a}	0^{a}	100^{b}	0^{a}	0^{a}	0^{a}	100^{b}	0^{a}	0^{a}	0^{a}	100^{b}	0^{a}	0^{a}	0^{a}	100^{b}
	28	0^{a}	0^{a}	0^{a}	–	0^{a}	0^{a}	0^{a}	–	0^{a}	0^{a}	0^{a}	–	0^{a}	0^{a}	0^{a}	–

Small superscript letters (a, b, c, d, e, f, g, and h) in the columns and the rows denote significant difference ($P < 0.05$).
HE, hexane neem seed extract emulsion; DE, dichloromethane neem seed extract emulsion; ME, methanol neem seed extract emulsion; AE, aqueous neem seed extract emulsion; C, control.

(concentration 5%) which resulted in 65% and 100% mortality of newly hatched larvae at 3rd and 5th days, respectively. Regarding 10% DE, it resulted in 75 and 100% mortality at 1st and 3rd days posttreatment, respectively, while at the concentration of 15%, death of all hatched larvae at 1st DPT occurred. In accordance with nymphs, as shown in **Table 7**, there was a significant effect of hexane extract and methanol extract on the mortality of *H. dromedarii* nymphs. In this regard, hexane extract at a concentration of 5% induced a mortality rate of nymphs reaching 80 and 100% at the first day posttreatment and 3rd day posttreatment, respectively. Furthermore, the mortality rate of nymphs reached 100% at concentrations of 10 and 15% of hexane extract at first day posttreatment. In the case of methanol extracts, the mortality of nymphs reached to 40 and 100% at the first day posttreatment and 3rd day posttreatment, respectively, while the mortality reached 100% at concentrations of 10 and 15% using the same extract on the first day posttreatment. On the other hand, the lowest effect was observed with dichloromethane, and no effect gained using the aqueous extract.

TABLE 6 | *In vitro* mortality rates of *Hyalomma* spp. larvae treated with 5, 10, and 15% of hexane, methanol, dichloromethane, and aqueous extracts emulsions of neem seeds.

DPT	Extracts and their concentration											
	5%				10%				15%			
	HE	DE	ME	AE	HE	DE	ME	AE	HE	DE	ME	AE
1	100^{a}	0^{d}	80^{b}	0^{d}	100^{a}	75^{b}	100^{a}	0^{d}	100^{a}	100^{a}	100^{a}	0^{d}
3	–	65^{c}	100^{a}	0^{d}	–	100^{a}	–	0^{d}	–	–	–	0^{d}
5	–	100^{a}	–	0^{d}	–	–	–	0^{d}	–	–	–	0^{d}
7	–	–	–	0^{d}	–	–	–	0^{d}	–	–	–	0^{d}
12	–	–	–	0^{d}	–	–	–	0^{d}	–	–	–	0^{d}
20	–	–	–	100^{a}	–	–	–	100^{a}	–	–	–	100^{a}

Small superscript letters (a, b, c, and d) in the columns and the rows denote significant difference ($P < 0.05$).
HE, hexane neem seed extract emulsion; DE, dichloromethane neem seed extract emulsion; ME, methanol neem seed extract emulsion; C, control; AE, aqueous neem seed extract emulsion.

TABLE 7 | *In vitro* mortality rates of *Hyalomma* spp. *nymphs* treated with 5, 10, and 15% of hexane, methanol, dichloromethane, and aqueous extract emulsions of neem seeds.

DPT	Extracts and their concentration											
	5%				10%				15%			
	HE	DE	ME	AM	HE	DE	ME	AE	HE	DE	ME	AE
1	80^{ab}	0^{f}	40^{c}	0^{f}	100^{a}	0^{f}	100^{a}	0^{f}	100^{a}	20^{d}	100^{a}	0^{f}
3	100^{a}	0^{f}	100^{a}	0^{f}	–	20^{d}	–	0^{f}	–	50^{bc}	–	0^{f}
5	–	10^{e}	–	0^{f}	–	20^{d}	–	0^{f}	–	50^{bc}	–	0^{f}
7	–	10^{e}	–	0^{f}	–	40^{c}	–	0^{f}	–	70^{b}	–	0^{f}
12	–	50^{bc}	–	0^{f}	–	40^{c}	–	0^{f}	–	80^{ab}	–	0^{f}
16	–	70^{b}	–	0^{f}	–	70^{b}	–	0^{f}	–	100^{a}	–	0^{f}
20	–	100^{a}	–	70^{b}	–	100^{a}	–	70^{b}	–	–	–	70^{b}
28	–	–	–	100^{a}	–	–	–	100^{a}	–	–	–	100^{a}

Small superscript letters (a, b, c, d, e, and f) in the columns and the rows denote significant difference ($P < 0.05$).
HE, hexane neem seed extract emulsion; DE, dichloromethane neem seed extract emulsion; ME, methanol neem seed extract emulsion; C, control; AE, aqueous neem seed extract emulsion.

DISCUSSION

Chemical acaricides have been considered the main tick control strategy in domestic animals (57). However, the inappropriate use of acaricides and their wider application resulted in an emerging problem of the development of tick resistance to these acaricides (16). Clearly, there is an urgent need for developing environmentally friendly, safe, effective anti-tick natural products that can interrupt the life cycle and all biological processes of insects and dispersal as a herbal acaricide (58, 59). The present study revealed potent acaricidal activity of different concentrations of neem seed extracts against different stages of *H. dromedarii* collected from camels, and their activity seem to depend on the used concentration. As shown in **Tables 1**–**4**, the immersion of different concentrations (5, 10, 15, and 20%) of hexane extract (HE) of neem seeds (neem oil) for 5 min resulted in 100% mortality of adult female ticks at 20, 5, 3, and 1 days posttreatment (DPT), respectively. Meanwhile, Butox® 5.0 and Diazinon-60 induced in mortality of all ticks at 3 and 5 DPT, respectively. Furthermore, application of the aqueous extract (AE) of neem seeds caused the mortality of adult ticks 43 DPT, which also occurred in the control groups. The same effect was also observed in previous studies on *Rhipicephalus microplus* (60, 61). Furthermore, another previous report (62) revealed that azadirachtin causes a significant increase in the mortality rate of unfed adults, which reached to 100% on 15th DPT. A previous study documented the potent activity of neem against eggs, immature and adult stages of *Hyalomma anatolicum excavatum* at concentrations of 1.6, 3.2, 6.4, and 12.8%. In the same study, a significant increase in the hatching rate was observed during the first 7 days posttreatment, followed by incompletely developed and dead larvae, and then after 15 days, neem resulted in hatching failure and induced a significant increase in mortality rates of newly hatched larvae, unfed larvae, and unfed adults (63).

As shown in **Tables 5**–**7**, the present study revealed that the application of HE induced hatching failure and a high acaricidal effect on oviposition, egg hatchability larvae, and nymphs. Similarly, Al-Rajhy et al. (64) investigated the effects of neem on *Hyalomma anatolicum* ticks and revealed a high acaricidal effect of azadirachtin at low concentrations against larvae and nymphs. Another previous study concluded that various concentrations (10%−100%) of neem seed oil were able to kill all *Boophilus decoloratus* larvae in cattle after a period of 24–27 h (65). By contrast, the obtained results disagree with those of a previous

study (64) which reported that azadirachtin had no effects on egg production with a significant reduction in the feeding activity of larvae and a 60% reduction in molting. Another previous study (62) documented the different effects of commercial neem seed oil (Neem Azal F) on *H. anatolicum excavatum* ticks that included an increased hatching rate and earlier hatching before the larvae were fully viable. As shown in our results (**Tables 1**–**4**), the used concentrations (5, 10, 15, and 20%) of the dichloromethane extract (DE) of neem seeds exhibited a low acaricidal effect on engorged adult ticks of *H. dromedarii* from the 5th day of application and continued up to an increase at 43th, 37th, 37th, and 28th DPT, respectively, resulting in 100% mortality. The possible explanation is the absence of azadirachtin in DE. Regarding the effect of DE against egg hatchability, it had a highly acaricidal effect on egg hatchability (**Table 5**) at all concentrations and a highly acaricidal effect on larvae at 15% concentration (**Table 6**), resulting in 100% mortality the 1st DPT. Similar effects were observed by Choudhury (66). The possible explanation might be attributed to the lethal effect of salannin compound (67). Salannin is one of the active components of neem with insect growth-regulating and antifeedant activity since it increases the larval stage duration and causes delayed molt, leading to decreased pupal weight that results in larval and pupal mortality (68). On the other hand, a low acaricidal effect of DE at 15% concentration was reported in nymphs, from the 1st day of application and continued up to 16th DPT, resulting in 100% mortality (**Table 7**). These findings are consistent with those of a previous study (62) which pointed out that DE contained a large amount of nimbin and salannin (69); nimbin had no significant effect on insects, but salannin had moderate antifeedant and growth-disrupting properties (67). Moreover, DE impaired oviposition at 5, 10, 15, and 20% concentrations by 87.5, 81.25, 75, and 68.75%. In addition, DE impaired the fertility by 100, 91.5, 75, and 68.25% at 5, 10, 15, and 20% concentrations, respectively.

Regarding the effect of methanol extract (ME) of neem seeds on ticks, which is shown in **Tables 2**–**4**, ME, at 10, 15, and 20% concentrations, exhibited a highly acaricidal effect against engorged adult ticks of *H. dromedarii* from the 1st day of application and continued up to 28th DPT, reaching 100% mortality. The present results are in agreement with those of a previous study which revealed the acaricidal effect of ME of neem leaves against *H. dromedarii* ticks. Moreover, all concentrations of ME had a lethal effect on oviposition and fertility, with a high acaricidal effect on egg hatchability without influence on hatching rate (**Table 5**). Our result is in agreement with that reported in several previous studies (41, 60, 70). Moreover, ME exhibited a high acaricidal effect at 10% concentration on larvae and nymphs from 1st DPT (**Tables 6**, **7**), which is consistent with some previous reports (62). Importantly, the present study showed that the AE of neem seeds at all concentrations (**Tables 1**, **5**–**7**) had no effect on the adult tick of *H. dromedarii*, egg hatchability, oviposition, larvae, and nymphs. These findings are in harmony with data reported by Tamirat et al. (61). This possible explanation of these findings could be attributed to the hypothesis that several polar compounds, like sugars and proteins, are eliminated in the aqueous extract (71). In the present work, the statistical analysis revealed a significant difference in the efficacy and effects of application of Butox (5%), diazinon, 10% hexane, and control group ($p < 0.05$), as well as between 10% hexane extract and Butox (5%) and diazinon ($p > 0.05$), while there is no significant relationship between Butox 5% and diazinon ($p > 0.05$). The tabulated data concluded that the efficiency of Butox 5% and diazinon was more than that of 10% hexane extract against infested ticks. Despite the fact that the data of our current study indicated that synthetic chemical insecticides were more efficient in controlling ticks than neem extract oil, the application of neem at higher doses on affected animals might offer many advantages for the control of ectoparasites without the risk of toxicity to them (72).

CONCLUSION

The study concluded that the *in vitro* application of neem extracts showed high efficacy against camel ticks. More importantly, the hexane extract exhibited a highly acaricidal effect on adult ticks of camels from the first day of application and continued up to 20 days after treatment, resulting in 100% mortality. The present data provide a platform for the development of environment-friendly, non-toxic, non-accumulating medicines against ectoparasites, which could be carried out in a large scale in animal farms.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of the Faculty of Veterinary Medicine, Aswan University, Egypt, and the Institutional Review approval Board Number is 2020/5.

AUTHOR CONTRIBUTIONS

AG, DH, AE, SK, MK, and AM designed the idea of the conception, performed the methodology, formal analysis, data curation and supervision, and revised the manuscript. AA, EE, NE, EH, ML, FE-G, and EKE participated in the methodology, formal analysis, data curation, and scientific advice. AG, AE, SK, EE, and EKE drafted the manuscript, prepared the manuscript for publication, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Case Report: Congenital tuberculosis in an aborted dromedary camel fetus

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Tuberculosis (TB) is a serious public health problem worldwide, especially in tropical developing countries. Nevertheless, reports on congenital TB in humans and animals are extremely rare. In this study, abortion was reported in an 8-year-old she-camel at the 9th month of gestation. The she-camel appeared healthy in clinical examination, had a good body condition score, normal appetite, and had no signs of respiratory disease and fever. The expelled placenta was dark red-colored, thickened, and edematous with multifocal to coalescing ecchymotic hemorrhages on the allantoic surface. The striking finding was multiple, white-yellow, solid nodular lesions in the fetal lung, the pleura, and the liver. On histopathology, typical granulomatous lesions were detected in the lung and the liver characterized by caseous necrosis surrounded by lymphocyte and macrophage infiltration and concentric layers of fibrosis. The Ziehl-Neelsen staining detected scarce acid-fast bacilli in lung and liver tissues. The DNA extracted from tubercular lesions from the lung and liver showed amplification of the IS6110 region of the *Mycobacterium tuberculosis* complex by PCR. The sequencing and phylogenetic analysis revealed a close association of these sequences with *Mycobacterium tuberculosis*. The she-camel was detected positive for a single intradermal tuberculin test performed 24 h after abortion. This is the first report on congenital TB caused by *M. tuberculosis* in a dromedary camel fetus with a possible vertical transmission.

KEYWORDS

camel, congenital tuberculosis, *Mycobacterium tuberculosis*, pathology, vertical transmission, abortion

Introduction

Tuberculosis (TB) is an ancient chronic contagious granulomatous disease having zoonotic and economic potential all over the world, especially in tropical developing countries. As per the WHO, 1.3 million deaths were reported as caused by TB globally in 2020, and TB mortality has been more severely impacted by the COVID-19 pandemic

than HIV/AIDS (1). In affected countries, the disease has an important socio-economic and public health-related impact and also represents a serious constraint in the trade of animals and their products (2). The emergence of multidrug-resistant TB is a global threat and a big challenge for effective control of the disease all over the world.

In dromedary camels, Mycobacteria belonging to the *Mycobacterium tuberculosis* complex (MTBC) have been frequently isolated; however, *Mycobacterium bovis* was reported as the most common etiological agent (3, 4). The most frequent clinical signs of camel TB are chronic weight loss, weakness, and lethargy; nevertheless, respiratory signs and fever were also recorded infrequently (3, 5). The lesions usually form in the lungs and the associated lymph nodes, and hematogenous or lymphatic spread can occur to the other organs (6).

The transmission of TB in camels may occur through contact with infected camels or other livestock and the route of infection are mainly through inhalation or ingestion (6). Although abortions and infertility due to TB lesions in the uterus have been sporadically reported in cattle (7), the intrauterine infection of TB is not yet reported in camels. This study describes a case of congenital TB caused by *M. tuberculosis* in an aborted dromedary camel fetus.

Case description

An 8-year-old Kachchi breed of dromedary camel was presented with abortion after the 9th month of gestation during her 2nd pregnancy. This camel belonged to a herd comprised of 350 dromedary camels in the Thar desert of Rajasthan, India. The herd is maintained in a semi-intensive husbandry system, mostly in outdoor facilities, and fed with a mix of pellet feed, hay, and *ad libitum* water. A history of sporadic occurrence of TB has been reported in this herd. Clinically, the aborted she-camel appeared healthy with a good body condition score and normal appetite and did not show any respiratory symptoms and fever at the time of the abortion. The she-camel was tested negative for brucellosis on Rose Bengal Plate Test. However, she tested positive in a single intradermal tuberculin test performed a day after abortion, showing marked swelling and a 2-fold increase in skin thickness at the injection site.

After the abortion, the placenta and aborted fetus were examined for gross lesions. The expelled placenta was thickened, edematous, and multifocal to coalescing ecchymotic hemorrhages on the allantoic surface (Figure 1A). The fetus showed generalized subcutaneous edema, congestion, and a moderate amount of sero-hemorrhagic fluid in the abdominal and thoracic cavity along with generalized congestion of all internal organs, which is likely due to autolysis and hemoglobin imbibition. The striking finding in the fetus was the presence of multiple, white-yellow, solid nodules scattered over the lung, the pleura, and the liver which measured from 2 to 15 mm in diameter (Figure 1B). The lung was collapsed, severely congested, and had multiple small white-yellow tubercle nodules scattered on all lobes. These nodules were also found attached to the pleura and inner surface of the rib cage. The liver was found enlarged considerably, congested, and had multiple tubercle nodules (Figure 1C). The other organs viz., the heart, the spleen, the kidney, and the intestines showed severe generalized congestion without any evidence of tuberculous lesions.

The tissue samples suspected to have TB lesions, such as the lung, the liver, and the placenta, were collected in 10% neutral-buffered formalin for histopathology, as well as in sterile vials for DNA extraction. For histopathology, tissues after fixation were embedded in paraffin, cut into 4-μm-thick sections and stained with hematoxylin and eosin. Selected sections were also subjected to Ziehl-Neelsen (ZN) staining. On histology, the lung and liver sections showed typical granulomatous lesions characterized by a central area of caseous necrosis and mineralization surrounded by scattered lymphocytes, macrophages, and/or occasional giant cells, and concentric layers of fibrosis (Figures 2A,B, 3A). Scarce acid-fast bacilli were observed on ZN-stained sections of the lung and the liver (Figure 3B). Histopathology of the placenta showed normal physiologic and/or autolytic changes of mineralization of the chorionic epithelium, necrosis of villous stroma, and hyperemic blood vessels. However, granulomatous inflammation and acid-fast bacilli were not observed.

Tissues (placenta, fetal lung, and liver) were processed for DNA extraction using the PureLink™ Genomic DNA Mini Kit (Invitrogen). The DNA was subjected to PCR amplification of a 245bp region of IS6110 sequence specific for the MTBC, using primer pairs INS1 (5′-CGTGAGGGCATCGAGGTGGC-3′) and INS2 (5′-GCGTAGGCGTCGGTGACAAA-3′) (8). Briefly, a 25 μl reaction was prepared using 12.5 μl Gotaq® green master mix (Promega), 1 μl each primer (10 picomoles), 5 μl of DNA, and 5.5 μl of nuclease-free water. The cycling conditions used were initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 1 min. This was followed by a final extension step of 72°C for 10 min. The PCR-amplified products were visualized in 2% agarose gel and the purified PCR products were subjected to nucleotide sequencing for the IS6110 gene using Sanger sequencing based on the chain-terminating dideoxynucleosides method (Eurofins, India). These sequences were deposited in NCBI GenBank (accession numbers: MW393780 and OL436218) and aligned with the published sequences for phylogenetic analysis using the ClustalW tool and the Maximum Composite Likelihood method (9). This analysis involved 30 nucleotide sequences, and the evolutionary analyses were conducted in MEGA X (10). The DNA extracted from the lung and the liver showed amplification of the IS6110 region of MTBC by PCR (Supplementary Figure 1). The sequencing and phylogenetic

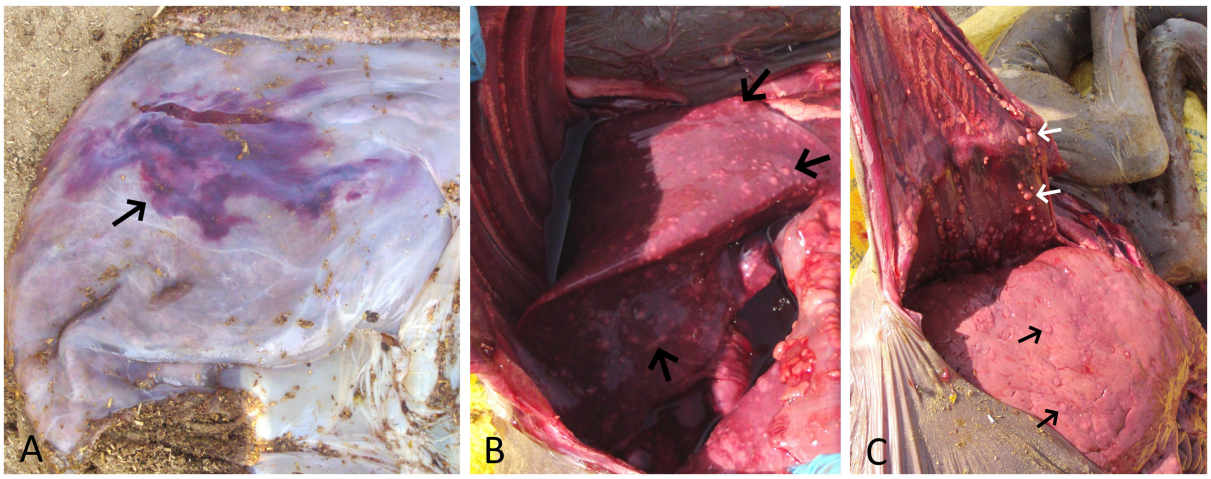

FIGURE 1
(A) Placenta showing edema and a large area of ecchymotic hemorrhage (arrow) on the allantoic surface. **(B)** Fetal lung showing congestion and multiple yellow-white tubercle nodules scattered throughout its surface (arrow). **(C)** Enlarged and congested fetal liver showing multiple yellow-white tubercle nodules (black arrow). Also, note multiple tubercle nodules adhered to the inner surface of the thoracic cavity (white arrow).

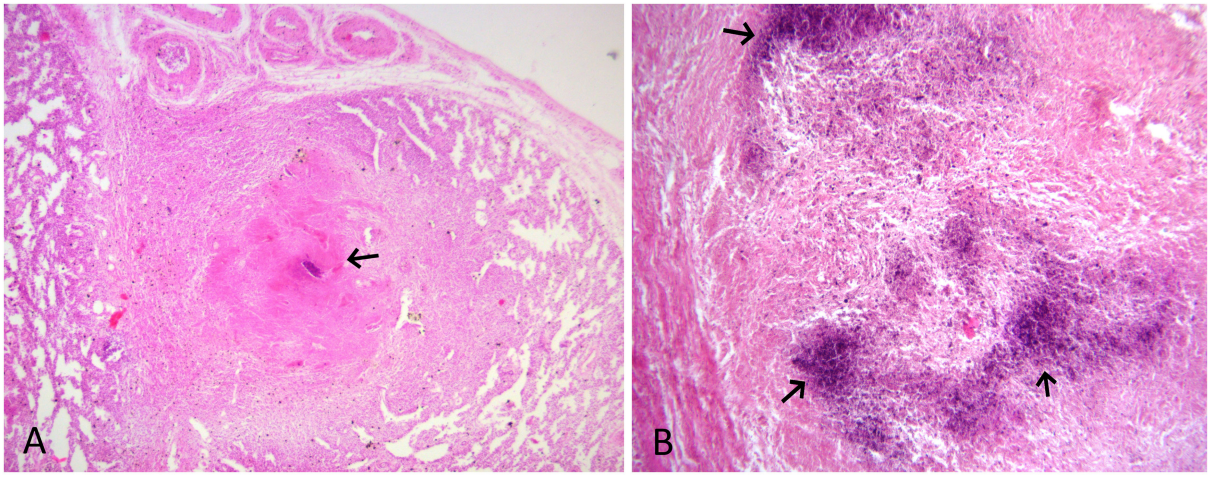

FIGURE 2
(A) HE-stained lung section showing granuloma with mineralization and caseous necrosis (arrow) surrounded by scattered lymphocyte and macrophage infiltration, and concentric layers of fibrosis. HE x 100. **(B)** HE-stained liver section showing dark blue areas of mineralization (arrow) and macrophage and fibrous tissue infiltration. HE x 200.

analysis revealed that sequences in this study clustered with *M. tuberculosis*.

On a managemental aspect, this she-camel was isolated and maintained away from the herd after abortion. After 1 year of isolation, the she-camel exhibited the clinical signs of chronic infection, including poor appetite, weakness, and progressive emaciation. Eventually, this she-camel died after 7 months of exhibiting symptoms. Considering the clinical history and the safety of the personnel, neither necropsy nor tissue evaluation was performed on this she-camel. Instead, the carcass was immediately disposed of by deep burying.

Discussion

Based on the pathological findings and detection of the MTBC genome from the fetal tissues, the case was etiologically diagnosed as fetal systemic mycobacteriosis caused by *M. tuberculosis* infection. The systemic TB lesions in aborted fetuses characterized by granulomatous inflammation were comparable with earlier reports of systemic mycobacteriosis in an aborted mare (11) and congenital TB in a newborn calf (12), suggesting the vertical transmission of TB bacilli from the infected dam to the fetus in the dromedary camel. This is known as the first

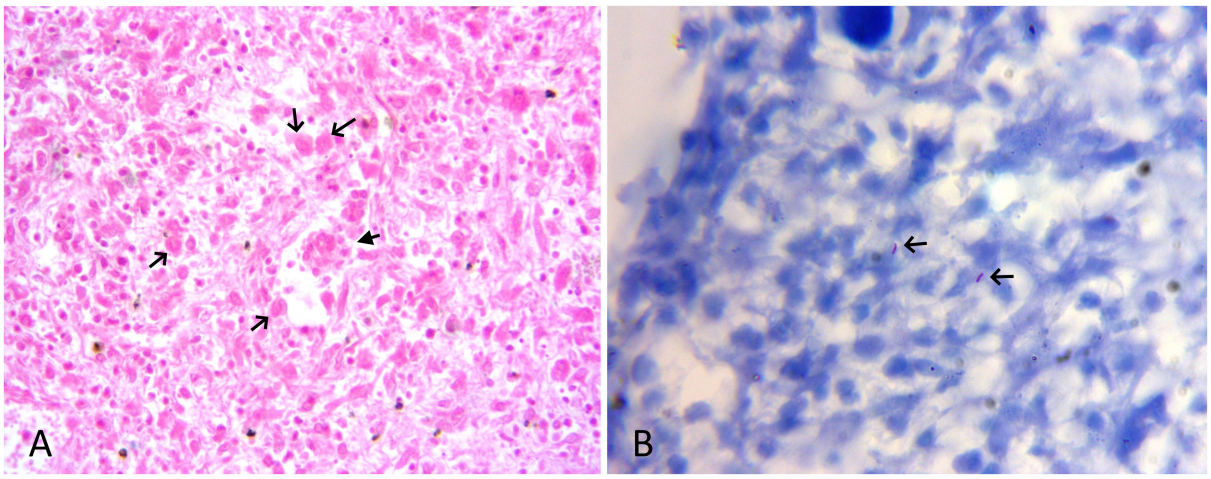

FIGURE 3
(A) HE-stained lung section showing scattered macrophages (black arrow), few lymphocytes, and a multinucleate giant cell (filled arrow). HE x 400. **(B)** ZN-stained section of liver showing sparse acid-fast bacilli (arrow). ZN x 1,000.

report of a congenital form of TB in camels. The congenital forms of the disease can occur when the disease involves the dam's genital tract or placenta (12). In such cases, TB bacilli are introduced into the fetus hematogenously *via* the umbilical vein, or *via* infected amniotic fluid ingested or aspirated *in utero* or at birth (13, 14). The significant lesions in the chorionic epithelium of the placenta can also be responsible for inadequate nutrition or fetal oxygenation resulting in fetal anoxia and abortion (15). However, granulomas or foci of caseous inflammation were absent in the placenta, which is intriguing. Nevertheless, the presence of mineralization, necrosis, cell desquamation, and minimal inflammatory cells observed in the placenta could be a resemblance to physiologic or autolytic changes observed in cattle placenta (16).

Although emaciation, weakness, and respiratory symptoms are commonly reported in TB-affected camels (3), abortion due to systemic fetal mycobacterial infection is not yet recorded. However, this she-camel exhibited no apparent clinical signs apart from abortion. It is possible that this she-camel was in the early stage of infection or had latent TB infection with minimal lesions and no apparent symptoms. As TB has a very long incubation period, physiological stresses, such as pregnancy and poor nutrition state could have triggered the activation of mycobacterial infection after abortion (17, 18). Moreover, the increased susceptibility to infections has been observed in periparturient cows mainly due to deficient systemic and local immune responses around parturition (18). It was suggested that bacterial invasion of the chorionic surface and subsequent hematogenous spread might be responsible for causing the fetus' systemic infection in equine species (11). Since camel placenta is epitheliochorial and resembles equine placenta, and hence this infectious route can also be possible in camels.

Since the clinical signs of TB in camelids often go unnoticed, and they are asymptomatic until the disease is advanced (17), therefore, nomadic people, who are in close association with the rearing and handling of camels are, at high risk of being infected. Given this, camel TB is a disease of concern from the point of its economic and zoonotic significance, especially in countries where camel has special cultural and economic importance. Few countries, such as Australia, some Caribbean islands, and parts of South America, eradicated bovine TB using a test-and-slaughter policy, which has drastically reduced the incidence of disease in both animals and humans (19). In addition, to control the TB spreading between camels and humans in endemic areas, the focus should be given to regular surveillance using rapid diagnostic tests for earliest case detection, segregation of the suspected animals, and educating the camel farmers about the risk of infection.

In conclusion, a congenital transmission of mycobacteria is evident in camels. Also, a camel could be a potential source of latent TB infection. Hence, regular screening of camels for mycobacterial infection is suggested for minimizing the risk associated with the spread of TB in endemic areas.

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 below: https://www.ncbi.nlm.nih.gov/genbank/, MW393780 and https://www.ncbi.nlm.nih.gov/genbank/, OL436218.

Ethics statement

Ethical review and approval was not required for the animal study because the case was natural infection and samples were collected from dead animal.

Author contributions

SN designed the experiments and prepared the manuscript. BJ, RR, VP, and SC collected the samples and reviewed the manuscript. AS supervised the project. All authors have read and approved the final version of the manuscript.

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Conflict of interest

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Knowledge, attitudes, and practices on camel respiratory diseases and conditions in Garissa and Isiolo, Kenya

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Background: Livestock farmers' attitudes, practices, and behaviors are major factors in infection prevention and control of animal diseases. Kenya has the fourth largest global camel population, and the industry has grown over the last two decades, transforming beyond the traditional camel-keeping areas to include peri-urban camel trade and value chain growth. The dromedary camel is resilient, and it is a preferred species in the arid and semi-arid areas (ASALs) of Kenya. However, it still faces many health and production challenges; to identify infection drivers and risky behaviors for camel respiratory illnesses and conditions in Kenya, we conducted a knowledge, attitudes, and practices (KAP) survey.

Method: Using a set of tools (questionnaires, key informant interviews, and focus group discussions), we interviewed camel owners, herders, agro-veterinary outlets, and other relevant value chain stakeholders in Garissa and Isiolo counties (n = 85). Data were analyzed using descriptive and analytic statistics.

Results: Most camel owners/herders are male and most are relatively uneducated (85.5%). The camels were used primarily for milk and meat production, income generation, and transport. Larger herd sizes (>30 camels) and owner/herder's lack of formal education are risk factors for owner-reported respiratory illnesses in camels. Major clinical signs of respiratory conditions were coughing (85.7%), nasal discharge (59.7%), and fever (23.4%). Diseases, lack of feeds, theft, and marketing challenges are the major constraints to camel production in Kenya. Owners-herders use drugs indiscriminately and this may contribute to antimicrobial resistance challenges.

Conclusion: Practitioners in the camel value chain want more commitment from the government and animal health officials on support services and access to veterinary services. Watering points, grazing areas, and marketing

points are the primary areas for congregating camels and have a significant potential for disease spread. Kenya camels have a massive capacity for rural and ASALs' livelihoods transformation but the identified health challenges, and other issues must be addressed. Further studies on the Kenyan camels' respiratory microbial ecology are important to understand microbial risks and reduce the burden of zoonotic infections. Intensification of risk communication and community engagement, and messaging targeted at behavior change interventions should be directed at camel value chain actors.

KEYWORDS

camel respiratory diseases, knowledge, attitudes, practices, Kenya, risk communication and community engagement

Introduction

The dromedary camel (*Camelus dromedarius*) is an important species in the Arid and Semi-Arid Lands (ASALs) agro-ecosystems of the world (1). Over 80% of the world's camel population lives in Africa with 60% of these in the Horn of Africa where they make a significant part of export, cross-border, and in-country trade, as well as food security and livelihoods of local communities (1–3). Additionally, the species is socio-culturally significant to some communities in matters, such as conflict resolution and dowry payment (4).

Kenya has the fourth largest camel population in the world, with an in-country estimate of 4.6 million in 2019 (5, 6). The camel industry in Kenya has grown steadily over the last two decades with the growth of peri-urban trade and expansion of camel keeping beyond the traditional areas (7, 8). The camel is becoming a preferred species for resilient livelihoods among pastoralist communities due to its superior adaptability to frequent droughts in the face of increasing climate variability (9).

This study was a follow-on from investigations into mass deaths of camels in northern Kenya and the greater Horn of Africa in early 2020. A respiratory syndrome characterized by nasal discharge, coughing, difficulty in breathing, and death affecting young camels had been reported in Marsabit, Wajir, Isiolo, and Garissa counties (10). The event raised speculations that the Middle East Respiratory Syndrome Corona virus (MERS-CoV), a zoonotic betacoronavirus, might have been the cause of the outbreak (11). Epidemiological and laboratory investigations, however, confirmed that it is a bacterial disease caused by Mannheimia haemolytica (10–12).

The emergence of human cases of MERS-CoV in Saudi Arabia in 2012, with subsequent evidence pointing to dromedary camels as a reservoir host for the virus, posed a threat to camel exports from the Horn of Africa to the Middle East (13–15). Growing evidence from phylogenetic studies on MERS-CoV isolates from the continent, however, shows that the lineages of the virus circulating in Africa are distinctly different from those circulating in humans and camels in the Middle East (16–19). This suggested that camel imports from Africa were not significant for the circulation of the virus in camels and humans in the Middle East (16).

The zoonotic potential of MERS-CoV clades circulating in Africa, however, remains a concern based on serological evidence of spillover of virus to humans at the camel–human interface and on infectivity studies, in tissue culture, of virus isolates from the region (19–22). The emergence of COVID-19 pandemic in 2019, caused by another betacoronavirus, the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-2), brought to the fore the need for accurate public information, education, and communication about camel respiratory conditions in relation to camel productivity and public health concerns. It is within this context that the Directorate of Veterinary Services, the County governments of Garissa and Isiolo, and the Food and Agriculture Organization of the United Nations (FAO) collaborated to undertake a Knowledge, Attitudes, and Practices (KAP) study on camel respiratory conditions among camel value-chain actors in the two counties. The purpose of the study was to provide data and evidence for the development of Information, Education and Communication materials (IECs) as part of communication interventions on camel respiratory conditions.

Materials and methods

A cross-sectional KAP survey was carried out in two counties of Isiolo and Garissa, Kenya (Figure 1) in the month of October 2020. The study involved camel owners, camel herders, community opinion leaders, and animal health professionals, as well as agro-veterinary shop owners. Several tools were employed in the study: questionnaires, key informant interviews, focus group discussions, and checklists for observations.

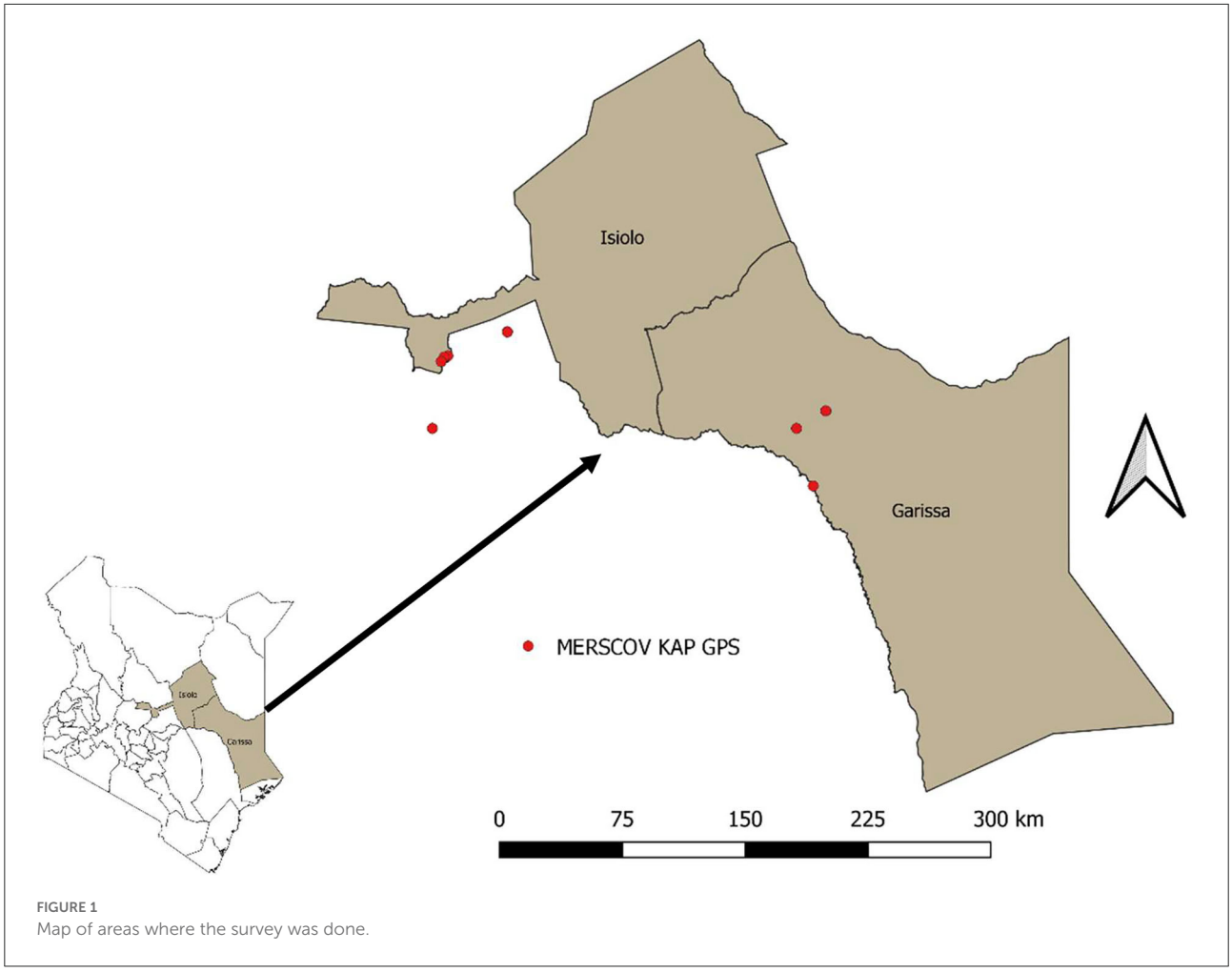

FIGURE 1
Map of areas where the survey was done.

Questionnaire development and administration

A questionnaire was developed through stakeholders' consultations and desk review. Specifically, responsible staff of the Directorate of Veterinary Services, Ministry of Agriculture, Livestock, Fisheries and Cooperatives (DVS MoALFC), and Food and Agriculture Organization of the United Nations (FAO) facilitated stakeholders meeting through which a list of issues that may play roles or influence respiratory disease incidences in camel were generated, through an iterative process, repetitive questions, and redundant issues were removed. The final list of questions was harmonized to produce a list of questions in the questionnaire (Supplementary material 1). This was pre-tested among five camel herders who did not form part of the interviewed participants. Based on the feedback, the questionnaire was adjusted, and the final version was used to conduct an interview in the field through administration to camel owners and camel herders. The questionnaire had three categories of respondents including camel owners, camel herders, and camel owners herding their own camels. The questionnaire was used to gather general information on camel health issues and specifically respiratory diseases. It was structured into four sections: demographics, knowledge, attitudes, and practices. The outputs were knowledge levels on the benefits of camels, constraints to camel keeping, general diseases and their causes, and clinical presentations of respiratory conditions (Supplementary material 1). The attitudes toward camel health issues were documented, and various practices among camel owners and herders were listed.

The second questionnaire developed was the Key Informant Interview (KII), which was directed to the animal health service providers including the County Veterinary Officers, Animal Health Assistants (AHAs), animal production officers, and agro-veterinary shop owners (Supplementary material 2). Camel traders were also interviewed based on their knowledge of camel health issues learned over time. In addition, Focus Group Discussions (FGDs) were held with four Camel Association groups using a semi-structured key informant

guide (Supplementary material 3). The questions in the Supplementary material 3 were aimed at triangulating the responses from the individual farmers and generating opinions on the relationship with the government, camel farming, welfare, and the challenges impacting camel farming in Kenya. Using these semi-structured tools, qualitative and quantitative data were collected. While the questionnaire survey provided a quantitative or numeric description of trends, attitudes, or opinions of the value chain stakeholders across the selected population, it also triggered some issues that needed some in-depth analyses. The key informant interviews provided the follow-up in-depth discussions with persons who were considered to have expert knowledge, in order to validate the earlier opinions. The focus group discussions were held to provide an open-ended cross-validations of the survey and to check whether the individual value chain perspective was similar or variant with the group views.

A total of 85 questionnaires were administered to camel owners and herders in Isiolo ($n = 44$) and Garissa ($n = 41$). Both counties were selected purposively from the list of counties with high camel populations (23). Villages were selected randomly from the list of villages per county. In Isiolo, the questionnaires were administered in Idafin, LMD, Bullo, Endomuru, Akadeli, Haidaffi, and Burrat villages. Similarly, in Garissa, the questionnaires were administered in the villages of Abdisamid, Shimbiry, and Bula-Rahma. In addition, 28 key informant interviews were carried out in the two counties. The participants were 16 veterinarians/animal health assistants (AHAs), 10 camel traders, one member of the Camel Association in Isiolo, and an official from the Livestock Market Trust (two opinion leaders). Four focused groups' discussions were held, two in Endomoru in Isiolo and another two in Bulla-Gawan, Garissa.

Data analysis

Data were entered into and filtered in Microsoft Excel v2016 (Microsoft Corporation, Redmond, Washington, USA). The data in the spreadsheet were transmitted into the IBM® SPSS® Statistics version 20 for analysis. Descriptive statistics including frequencies and exact confidence intervals at a 95% level were calculated. The leading constraint to camel production was determined using serial positioning. To determine the association between different variables chi-square tests were performed with a *p*-value set at 0.05. A pairwise correlation was determined among relevant variables with a significant association set at 0.05. Risk-based (sub-population level and population level risks in percentages) and odds-based (conditional maximum likelihood estimate of Odds Ratio) estimates of variables were carried out using the two-by-two table in OpenEpi® (24).

TABLE 1 Demographic variables of the respondents.

Demographic variable	Number	Percentage (%)
Gender	**85**	**100**
Male	71	83.5
Female	14	16.5
Age of respondents	**85**	**100**
18–25	7	8.2
26–35	21	24.7
36–45	25	29.4
46–55	14	16.5
56–65	8	9.4
66>	10	11.8
Education level of respondent	**85**	**100**
None/never been to school	66	77.6
Primary incomplete	7	8.2
Primary complete	8	9.4
Secondary incomplete	2	2.4
Secondary complete	1	1.2
Tertiary incomplete	1	1.2
Religion of respondent	**85**	**100**
Christian	5	5.9
Muslim	80	94.1
Herd size	**85**	**100**
Small (1–5 camels)	7	8.2
Medium (6–30 camels)	26	30.6
Large (>30 camels)	52	61.2
Respondent type	**85**	**100**
Owner	52	61.2
Herder	15	17.6
Both owner and herder	18	21.2

Bold values mean cumulative here.

Results

Demographics

Out of the 85 individual respondents, 71 (83.5%) were males and 14 (16%) were females. Most of the respondents identified as Muslim (94.1%) while the remaining 5.9% identified as Christian. A total of 70.6% of the respondents were in the age category of 26–55 years. The age distribution of the other respondents is indicated in Table 1. The majority of the respondents had not received a formal education, with 77.6% not having attended school, and an additional of 8.2% having not completed primary school. In terms of herd size, 61.2% of those interviewed had more than 30 camels (classified as large herd), 30.6% have medium herd sizes (6–30 camels), and only 8.2% have small herds (1–5 camels). Furthermore, the majority of the respondents were camel owners (61.2 %), and 17.6% were

TABLE 2 Risk and odds-based estimates of respiratory illnesses and conditions in Kenya camels.

Variable		Respiratory condition present	Respiratory condition absent	Sub-population level risk (%)	Population-level risk* (%)	CMLE Odds ratio**	*P*-value
Gender	Male	65	6	91.6	91.7	0.90 (0.03; 6.89)	1.00
	Female	12	1	92.3		1.00	NA
County	Isiolo	40	4	90.9	91.7	0.81 (0.14; 4.18)	0.81
	Garissa	37	3	92.5		1.00	NA
Herd size	Small	7	0	NA	90.9	–	–
	Medium	22	4	84.6		0.35 (0.06; 1.82	0.21
	Large	48	3	94.1		1.00	NA
Education	No formal education/ incomplete primary	67	5	93.1	91.7	2.64 (0.32; 15.46)	0.32
	Complete primary up to tertiary	10	2	83.3		1.00	NA
Responsibility to camel	Owner	46	5	90.2	90.9	0.66 (0.02; 5.29)	0.78
	Herder	14	1	93.3		1.00	NA
	Owner–herder	17	1	94.4	91.3	0.55 (0.02; 4.30)	0.65

*Risk-based estimates.
**Odds-based estimates.
CMLE, Conditional maximum likelihood estimate of Odds Ratio.
Based on the feedback from the respondents, the outcomes of respiratory conditions or diseases in the camel herds may lead to recovery (14.3%), death (41.5%), or uncertain situation of death or recovery (44.2%). NA, Not applicable.

purely camel herders, while the remainder 21.2% herded their own camels (Table 1).

Using the risk and odds-based estimates, the population-level risk for respiratory conditions in the studied Kenya's camel is 91.7%. The risk of respiratory conditions in the male camel (91.6%) is slightly less than in the female (92.3%) although the odds of the risk is 0.90 in male vs. female ($p = 1.00$). Similar profiles exist for differences between Isiolo (90.9%) and Garissa (92.5%) counties (OR = 0.81; $p = 0.81$). The medium-sized herd is 3-fold less likely and has a 9.5% less risk of contracting respiratory conditions ($p = 0.21$) (Table 2). Camel herds of individuals with no formal or incomplete primary education are 3-fold more likely and have 9.8% more risk of respiratory conditions compared to those who have completed primary schooling or more ($p = 0.32$). Compared to the herds managed by herders, herds of owners and those of individuals who combined the role of owner-herder are ~0.66-fold ($p = 0.78$) and 0.55-fold ($p = 0.65$) less likely to have respiratory conditions, respectively (Table 2). Based on the feedback from the respondents, the outcomes of respiratory conditions or diseases in the camel herds may lead to recovery (14.3%), death (41.5%), or uncertain situation of death or recovery (44.2%).

Using serial positioning, camel diseases were ranked as the greatest constraint to camel production, followed by feeds, marketing, and then theft (Table 3). Other issues that flagged up as constraints were predation, water scarcity, injuries and accidents to animals, poor farming and management system, cost of maintaining the herders, hardship experienced with herding, drought, and land disputes in that order.

TABLE 3 Constraints to camel production.

Constraints	Position 1 (%)	Position 2 (%)	Position 3 (%)	Position 4 (%)
Diseases	**53.6**	21.9	16.3	14.3
Feeds	14.3	**37.9**	16.3	0
Theft	17.9	21.9	12.2	**14.3**
Marketing	4.8	9.6	**30.6**	21.4

Bold values mean priority selection here.

Knowledge and awareness

The respondents confirmed that camel farming and management are beneficial and the lead reason why they farmed camel include the following: provision of milk (92.9%), meat (76.5%), income (72.9%), transport (34.1%), and for cultural activities for example during dowry payment (24.7%) (Figure 2A).

In terms of causes of diseases in camels, although the owners and herders were not able to mention specific diseases, they were aware of the causes of diseases in camels based on interactions with their animal health officials. Pests (mosquitoes, tsetse flies, and ticks) were reported by 77.6% of the respondents,

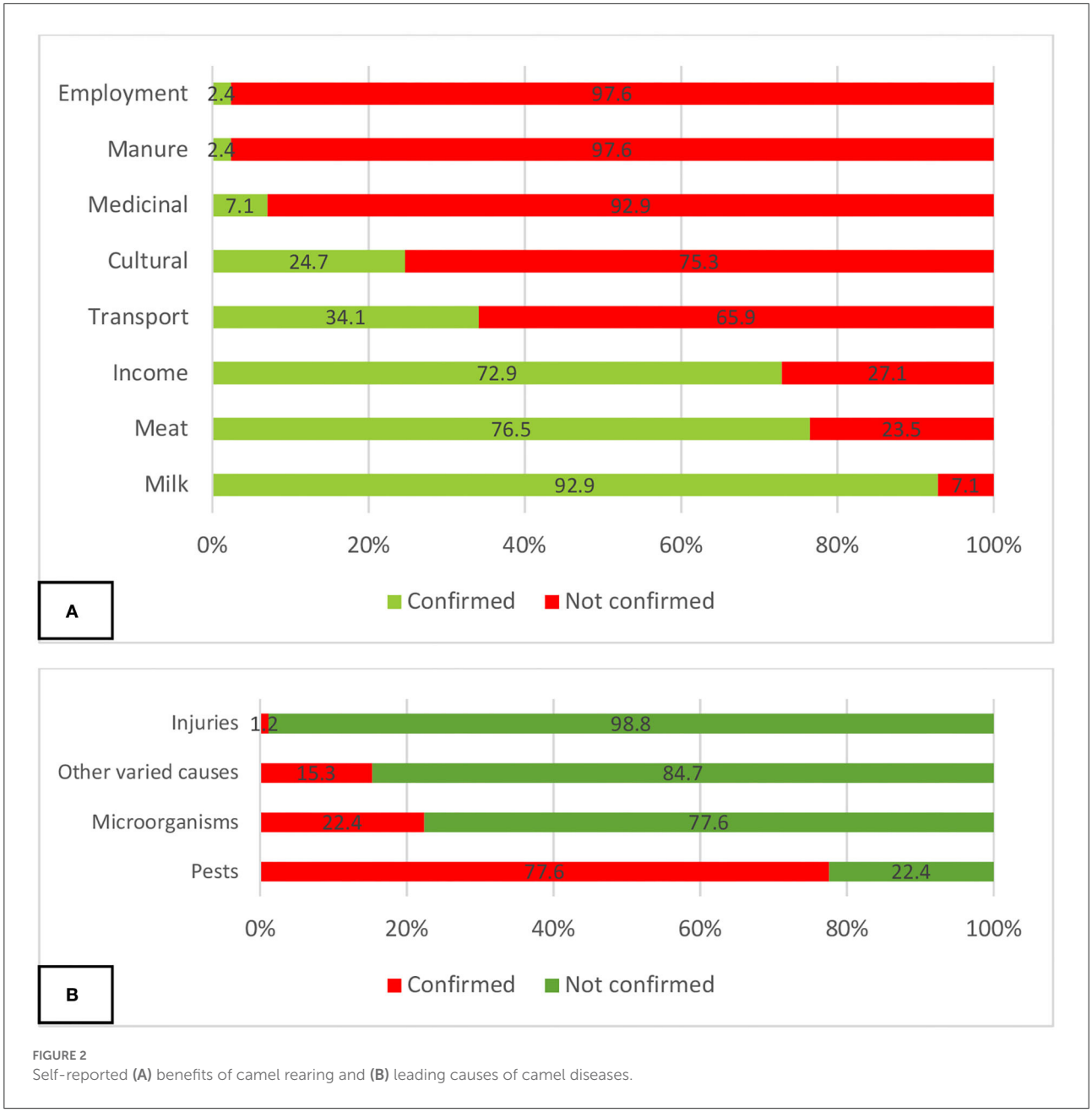

FIGURE 2
Self-reported **(A)** benefits of camel rearing and **(B)** leading causes of camel diseases.

microorganisms by 22.4%, injuries by 1.2%, and other causes mentioned by 15.3% (Figure 2B).

The majority of the respondents (90.6 %) reported that their herds had suffered from respiratory conditions, especially during the rains. The following clinical signs have been observed as predictors of respiratory conditions and diseases in camels: coughing (85.7%), nasal discharge (59.7%), fever (23.4%), loss of appetite (20.8%), and enlarged lymph nodes (19.5%) as the most common signs as listed by camel keepers in the two counties (Table 4). Other signs and symptoms included body weakness (15.6%), weight loss (14.3%), recumbency (11.7%), drop in milk production (9.1%), excessive lacrimation (tears) (9.1%), sneezing (7.8%), enlarged abdomen (6.5%), shivering (6.5%), difficulty in breathing (5.2%), sudden death (2.6%), abortion (2.6%), and foaming in the mouth (1.3%) (Table 4). Difficulty in breathing was moderately positively correlated with foaming in the mouth ($p < 0.05$). Weak positive correlations were observed between a drop in milk production and abortion; recumbency and foaming in the mouth; inappetence and sudden death; enlarged lymph nodes and excessive lacrimation; excessive lacrimation and foaming in the mouth; weight loss and drop in milk production; weight loss and fever; shivering and sudden

TABLE 4 Symptoms and clinical signs as predictors of respiratory diseases in camels.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
A	1.000																
B	−0.125	1.000															
C	0.013	−0.008	1.000														
D	0.123	0.185	−0.179	1.000													
E	0.067	0.088	0.021	0.030	1.000												
F	−0.039	−0.194	0.116	0.124	−0.039	1.000											
G	−0.044	0.010	0.208	−0.185	0.095	−0.061	1.000										
H	0.092	0.043	0.223	−0.058	**0.490***	−0.019	**0.315***	1.000									
I	0.161	−0.023	0.169	**0.298***	0.128	−0.053	0.023	**0.362***	1.000								
J	**−0.445***	0.037	0.244	−0.113	−0.098	0.165	−0.153	−0.048	−0.133	1.000							
K	0.102	0.099	0.122	−0.000	−0.063	−0.044	0.066	−0.031	−0.086	0.040	1.000						
L	0.013	0.049	0.128	−0.127	−0.104	−0.072	0.175	−0.051	−0.015	−0.078	−0.117	1.000					
M	0.161	**−0.305***	−0.167	0.069	−0.076	−0.053	0.023	−0.037	−0.103	−0.133	0.098	−0.015	1.000				
N	0.013	0.061	**0.318***	−0.083	−0.039	−0.027	0.194	−0.019	−0.053	−0.069	**0.288***	−0.072	−0.053	1.000			
O	−0.216	−0.023	0.169	−0.160	−0.076	**0.231***	0.164	−0.037	−0.103	**0.385***	−0.086	−0.015	−0.103	−0.053	1.000		
P	**−0.371***	0.109	−0.154	−0.147	−0.070	−0.049	−0.109	−0.034	−0.095	−0.122	−0.079	−0.129	−0.095	−0.049	−0.095	1.000	
Q	−0.131	−0.081	0.012	−0.125	−0.133	−0.093	−0.111	−0.065	−0.073	**0.297***	−0.025	−0.075	0.142	−0.093	0.142	**0.295***	1.000

A, Nasal discharge; B, Coughing; C, Inappetence; D, Enlarged lymph nodes; E, Difficulty in breathing; F, Abortion; G, Recumbency; H, Foaming in the mouth; I, Excessive lacrimation (tears); J, Weight loss; K, Shivering; L, Body weakness; M, Enlarged abdomen; N, Sudden death; O, Drop in milk production; P, Sneezing; Q, Fever.
*p-value ≤ 0.05. Bold values mean significant here.

death; as well as sneezing and fever ($p < 0.05$). However, a moderate negative correlation was observed between nasal discharge and weight loss, but a weak negative correlation was observed between nasal discharge and sneezing and between coughing and an enlarged abdomen ($p < 0.05$) (Table 4).

Attitudes

Camels were considered to be hardy animals by 58.8% of the respondents, while 41.2% thought not. Respiratory diseases were thought to be common in young camels by 60% of respondents, 30.6% thought it was common in all ages while 23.5% thought it was common in older camels (Table 5). On seasonality of the occurrence of respiratory diseases, most respondents (56.5%) reported that they occur more during the rainy season. While others thought that it occurs in dry and cold seasons; 35.35 and 29.4%, respectively. Others thought it occurs throughout the year (11.8%) while 3.5% thought respiratory diseases were common during cultural occasions (Table 5). The community disease reporters and the animal health assistants were more available than the veterinarians to handle camel health. According to respondents, herbalists and elders, community disease reporters, and animal health assistants were the preferred health service providers for their camels (Table 5). In addition, various sources of information and indigenous knowledge on camel health exist, and interpersonal information sharing and radio remain the leading sources of information. The interpersonal channel typically occurs at the watering points, by elders, at the markets, and places of worships (Table 5). Camel owners and herders perceived that government support for camel production is wanting (64.7%) although 29.4% believed that there is some government support.

Practices

When camels fell sick, the most preferred practice was treatment, first by the owners (71.8%). Only 21.2% of the respondents consulted an animal health service provider. Others prayed for the camels (1.2%) or did a variety of other things (5.8%). Using serial positioning to analyze this practice, the treatment by owners, isolation of sick camels, let them recover on their own, seek help from herbalists, and slaughter of sick animal were practiced in this descending order (Table 6). When faced with the challenge of feed scarcity most camel keepers migrate in search of pastures (88.4%), 7.0% buy feeds, 2.3% rent pasture fields, and 2.3% do nothing. To address water scarcity, camel farmers migrate to areas with watering points or do nothing. To overcome marketing challenges the majority (70%) sell camels at low prices, others seek government support, look for alternative markets or do nothing about it. To address the challenge of theft, camel farmers report to government authorities (41.2%), 35.3% attempt tracking and retrieval by self, and 8.8% fight back while others migrate, brand their animals, keep guard, or do nothing. Watering points, grazing, marketing,

TABLE 5 Attitudes and disposition to camel diseases and information sources.

Variable	Classification	Frequency	Percentage
Hardiness of camel	Yes	50	58.8
	No	35	41.2
Age predisposition for respiratory diseases ($n = 85$)	Young camels	51	60.0
	All ages	26	30.6
	Old camels	20	23.5
	Lactating	8	9.4
	Pregnant	6	7.1
Seasonal predisposition to respiratory diseases ($n = 85$)	Rainy season	48	56.6
	Cold season	30	35.3
	Dry season	25	29.4
	All the year round	10	11.8
	More during cultural occasion	3	3.5
Availability of animal health officials ($n = 84$)	Community disease reporter*	22	26.2
	Animal health assistant	21	25.0
	Veterinarian	14	16.7
Preferred service provider ($n = 83$)	Herbalist/elders	24	28.9
	Community disease reporter	20	24.1
	Animal health assistant	14	16.9
Level of government support ($n = 85$)	No support	55	64.7
	Little support	25	29.4
	Sufficient support	3	3.5
	I don't know	1	1.2
Preferred source of information ($n = 83$)	Interpersonal communication with fellow herd owner	24	28.9
	Radio	16	19.3
	Herbalist	11	13.3
	Agro-veterinary shop owner	7	8.4
	Community opinion leader	6	7.2
	Chief "*barazas*"**	5	6.0
	Veterinarian/animal health assistant	4	4.8
	Mobile phones	3	3.6
	Self-motivated learning	2	2.4
	Community disease reporter	2	2.4
	Training	1	1.2
	Farmers' group	1	1.2
	No preference	1	1.2

*Community disease reporters are community animal health volunteers who are not officially remunerated for their services but may be paid tokens by the community for their services.
**Baraza is the informal village-level dissemination fora.
Attitudes that may increase risk perception to camel respiratory diseases: (1) Association of climatic conditions (cold, dry, and rainy) conditions to respiratory diseases, (2) Association of age to camel respiratory conditions, and (3) Perception that camels are highly valued animals.
Attitudes that may decrease risk perception to camel respiratory diseases: (1) Perception that camels are hardy animals, (2) Perception that government does not care for camels, (3) Low preference given to animal health professionals as a preferred source of information, (4) Doing nothing when encountered with challenges, and (5) Self-treatment of the sick camel.

congregating of camels for security purposes, migration, and during clashes were listed as occasions that camels from different herds meet (Table 6).

Discussion

The study was carried out on the camel value chain in Kenya with respect to the anthropological context (human activities) of camel owners and herders and how these influence camel respiratory diseases and conditions spread, prevention, and control. The camel industry is male-dominated, possibly due to the cultural settings in the two counties, patriarchy in raising large animals or other unknown considerations (25, 26). Such male domination has also been seen elsewhere in Africa (27, 28). Although women play significant roles in milking, milk handling and processing, and many other routine management practices, and may contribute a large chunk of household incomes,

TABLE 6 Practices associated with camel management in Kenya.

Variable	Classification	Frequency	Percentage
What is the most-preferred method of treating a sick camel? ($n = 85$)	Owner treat first	61	71.8
	Consult animal health service provider	18	21.2
	Pray for the camel	1	1.2
	Do a combination of practices	5	5.8
What do you do during feed scarcity? ($n = 85$)	Migrate in search of pasture	75	88.4
	Buy feeds	6	7.0
	Rent pasture field	2	2.3
	Do nothing	2	2.3
How do you overcome marketing challenge? ($n = 83$)	Sell at lower price	58	70
	Seek government support, look for alternative markets or do nothing	25	30
To address challenges of theft, what do you do? ($n = 85$)	Report to government authorities	35	41.2
	Attempt self-tracking and retrieval	30	35.3
	Fight back the invaders	7	8.8
	Others: Migrate to safer areas, brand animals, keep guard or do nothing.	13	14.7
		Serial position	
Ranking and positioning of treatment practice	Treatment by owners	1st	
	Isolation of sick camels	2nd	
	Allow the camel to recover on its own	3rd	
	Seek help from herbalists	4th	
	Slaughter of sick animal	5th	
List places where camels from different herds meet and interact ($n = 85$)	Watering points (90.6%), grazing (57.6%), marketing (36.5%), congregating of camels for security purposes (3.5%), migration (3.5%), and during clashes (2.4%)		

their roles may have been downplayed by the observed male domination of the industry (25, 28). In Kenya, other studies have been carried out among camel-keeping communities including KAP for Rift Valley fever (29), brucellosis among nomadic pastoralists and non-pastoralists (30), a review of zoonotic pathogens of dromedary camels and humans (31), and for hygiene associated with camel milk among handlers (32, 33), as well as in other neighboring countries (34).

This study revealed that the major reason for keeping camels is for purposes of milk and meat production, and for income generation. This confirms previous findings that camels contribute significantly to food and nutritional security in the ASALs of Kenya (35). Almost 86% of the respondents did not complete primary education. This low literacy level within the study population is worrisome because health-related messaging by public and animal health professionals is largely literal and may not achieve its aims among these populations. It is advocated that risk communication and community engagement interventions should make use of simple pictorial representation among camel pastoralists (36). The communities have rich indigenous knowledge of camel health, based on experience garnered over time, and socialization. Interpersonal channels of communication were also identified as the most preferred source of information. It is unnecessary to discard such information. Rather, this should be utilized to improve behavioral change intervention among camel pastoral communities (37). More work needs to be done to understand the most effective forms of communication, whether pictorials will work best, or whether radio and personal messaging using community animal health workers will achieve better results.

Whereas the camel owners and herders perceived that camels are hardy animals and are hardly susceptible to diseases, the population-level risk for respiratory infections and conditions among the study camel herd was 91.7%. This perception among a significant proportion of the community (58.8%) can negatively affect the health-seeking behavior of camels by their keeper. It also has the potential to delay timely medical intervention for sick camels. Theory and empirical evidence have demonstrated that perceptions of risk play a key role in motivating people to adopt healthy behaviors (38–40). People who are positively optimistic are likely to have a lower risk perception index and consider themselves at a lower risk of a disease outcome (41). They are thus unlikely to seek medical attention. This could also apply to camel farmers/keepers. The communities perceive camels as a neglected domestic animal by the government and that government veterinary services are

out of reach for most of them. This finding further reduces effective response by the camel owners. This also explains the administration of antimicrobials by the camel keepers instead of seeking for professional assistance on animal health from veterinarians.

Although not statistically significant, lack of education, large-sized herd, and being a herder posed risk of infection with respiratory conditions to camels. These factors as well as poverty have been identified as significant risks in zoonotic infections to humans and animals (42, 43). Associated with these findings, a variety of diseases were identified as the most important constraint to camel value chain development in parts of Kenya, and the lead cause of those diseases was pests and microorganisms. This finding is quite relevant in view of the challenges of accessing animal health services by these herders and camel owners. It has previously been reported that the diagnosis and treatment of sick animals by the owners and herders is practiced widely among the pastoralist communities, similar to the findings in our study (44). Seeking the assistance of herbalists, community disease reporters and occasionally animal health service providers was also common, and a few of the pastoralists reported slaughtering sick camels as a last resort. Furthermore, a significant number of camel keepers "*do nothing*" in response to animal health challenges. A "*do nothing*" response probably shows apathy, ignorance, or genuine discouragement due to a lack of support as far as camel health is concerned. It is plausible that these practices mentioned in the study were rampant because accessing professional veterinary services were difficult for these camel owners and herders.

The perceived hardiness of camels, which may delay reporting as explained above, may be associated with inconsistent clinical signs (e.g., coughing was observed in 85.7%, nasal discharge in 59.7%, fever in 23.4%, loss of appetite in 20.8%, and enlarged lymph nodes in 19.5% of the cases in camels, with variation across individuals and villages). Although we did not observe any consistent pattern (pathognomonic sign) with regard to these observed signs, perhaps, a clear categorization with regard to the signs and symptoms may have prevailed if these respiratory conditions were disaggregated by age, gender, and physiological conditions. It is also noted that most respiratory conditions present as respiratory complexes which may involve a number of respiratory pathogens (45, 46). As observed by the respondents, a high prevalence of respiratory diseases was associated with rainy and cold seasons and younger camels. Gardner et al. (47) have earlier confirmed the effect of these seasons on camel respiratory diseases. It should be noted that most of these camels are not housed in a proper shelter and are therefore exposed to inclement weather, especially during the rains and cold seasons, and the young animals are more affected by these conditions because they are likely to be more hypothermic and susceptible to physiologic stress (48, 49). This disposition that the extremes of weather are inimical to animal respiratory health is a positive finding because it can increase the risk perception of camel respiratory diseases and provide the basis for mitigation (50). Such positive views can be reinforced and linked with improved risk perceptions and knowledge of the importance of early diagnosis and treatment by veterinary practitioners.

Elders and herbalists were the most preferred source of camel health information based on respondents' feedback. It becomes relevant for animal health services providers (veterinarians/animal health assistants and agro-veterinary shop owners) to partner with these primary sources of information to disseminate information on risks, animal health, and good farming practices using local languages and community radio stations. Such partnerships may trigger behavior change intervention in animal health services in the ASALs.

Some of the identified practices associated with camel management in the ASALs of Kenya are important considerations for the improvement of the camel value chain, public and animal health management, and human conflict resolution. Firstly, owners treat sick animals first before consulting animal health service providers. This has the implication for the abuse of antimicrobials with potential passage to the human food chain. In addition, during feed scarcity or security challenges, most herders prefer to migrate in search of pasture. This particular practice has significant potential for herders—crop farmer conflicts, an issue that has been identified regularly in sub-Saharan Africa (51–53). In addition, some farmers do nothing or sell such sick camel at lower prices. It is likely that such camels may be slaughtered and served to humans and may introduce zoonotic or food-borne diseases to humans. Thirdly, owners–herders' attempts at self-tracking and retrieval of rustled or stolen camels and the practice of fighting back invaders are long associated with animal rustling, with unnecessary wasting of human lives. It becomes necessary that service delivery for crime reportage should be brought closer to these communities to reduce potential human conflicts associated with securing the stock. Finally, a number of high-risk areas have been identified including the watering points, the grazing areas, and the markets. The provision of necessary infrastructure services such as water, designated grazing areas, and bio-secure markets will positively impact on reducing the burden of camel diseases in the ASALs.

Conclusion

We have identified relevant knowledge, attitudes, perceptions, and practices of camel owners and herders on camel health. These identified knowledge, attitudes, and practices should serve as entry points in creating attitudinal and behavioral change in camel health.

Similarly, the animal health authorities should strive to be more responsive to the needs of camel pastoral communities in Kenya to reduce the potential burden of zoonoses and food-borne illnesses associated with camel. Development of specific communication strategy that targets the camel pastoralist communities is recommended for implementation.

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 study of human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

Concept: ET, MA, TN, SV, and EG. Fieldwork: JO, SM, BM, PK, BA, ET, and TN. Analysis: FF, SM, TN, and ET. Project administration: ON, MA, JM, HH, FF, and SV. Contributed resources: ON, MA, SV, JM, LM, HH, TN, and FF. Writing of initial draft: JO, SM, MA, ET, TN, and FF. All authors writing and review of the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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The influence of different examiners on the Body Condition Score (BCS) in South American camelids—Experiences from a mixed llama and alpaca herd

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Particularly in unshorn llamas and alpacas with a dense fiber coat, changes in body condition often remain undetected for a long time. Manual palpation of the lumbar vertebrae is hence a simple and practical method for the objective assessment of body condition in South American camelids (SAC). Depending on tissue coverage, a body condition score (BCS) of 1 (emaciated) to 5 (obese) with an optimum of 3 is assigned. To date, there is a lack of detailed information on the comparability of the results when the BCS in llamas or alpacas is assessed by different examiners. Reliability of BCS assessment of 20 llamas and nine alpacas during a veterinary herd visit by six examiners was hence evaluated in this study. A gold standard BCS (gsBCS) was calculated from the results of the two most experienced examiners. The other examiners deviated by a maximum of 0.5 score points from the gsBCS in more than 80% of the animals. Inter-rater reliability statistics between the assessors were comparable to those in body condition scoring in sheep and cattle ($r = 0.52–0.89$; $\tau = 0.43–0.80$; $\kappa_W = 0.50–0.79$). Agreements were higher among the more experienced assessors. Based on the results, the assessment of BCS in SAC by palpation of the lumbar vertebrae can be considered as a simple and reproducible method to reliably determine nutritional status in llamas and alpacas.

KEYWORDS

emaciation, clinical score, inter-rater reliability, nutrition, herd management, endoparasitosis, camelids

1. Introduction

The husbandry of South American camelids (SAC) is becoming more and more popular in Europe (1–4). In case of disease, llamas and alpacas are, however, often presented late for veterinary care. Hence, the animals are often severely emaciated or reveal anemia (5). In a recently published evaluation of 300 SAC presented to our clinic, we found that 60% of the alpacas and 70% of the llamas revealed a Body Condition Score (BCS) lower than the optimal score of three (5). At the same time, half of the SAC farms in Germany that participated in an online survey recently stated that they never had problems with emaciation. Furthermore, a quarter observed <1 case of emaciation per year (1). This survey also showed that the occurrence of gastrointestinal endoparasitic infections and emaciation was more likely on farms with more animals than those with fewer animals (1). This discrepancy between the high amount of emaciated animals that are presented to the clinic and a rather low awareness of emaciation on the farms indicates that the assessment of the nutritional status is of particular importance in husbandry of SAC to recognize emaciation in time. Inadequate feeding management, chronic

diseases, dental problems, and especially gastrointestinal endoparasites can lead to a poor nutritional status related to a low BCS (6–8). The decrease in body condition, sometimes within a relatively short space of time, is overlooked by the keeper due to the animal's dense fiber coat. In addition, SAC generally hide symptoms of disease for a long time and only display them at a very late stage (6). Visual examination alone is hence insufficient and may lead to incorrect results. When assessing the nutritional status of llamas and alpacas, manual palpation is vital (9). For the standardized assessment of the nutritional status in SAC, descriptions of a body condition score (BCS) from previous studies are available. Most of the authors recommend the palpatory examination of the lumbar spine for determining the BCS in SAC (9–16). However, depending on the source, other body regions, such as the thorax behind the elbow, the paralumbar fossa, or the area between the front and rear legs, are sometimes included in the assessment of the BCS of llamas and alpacas (6, 10, 12). In cattle, where the concept of body condition scoring is an important tool in herd management (17), several studies on the learnability and reproducibility of the BCS are available (18–21). Similar data can be found for sheep (22–24). To the best of our knowledge, accurate data on the comparability of BCS in llamas and alpacas are currently unavailable. In order to investigate the inter-rater reliability (25) for the BCS in SAC by palpation of the lumbar spine, we evaluated the results of six examiners with different levels of expertise assessing the BCS of llamas and alpacas during a herd visit in northern Germany.

2. Material and methods

2.1. Herd

The mixed llama and alpaca herd was located in northern Germany and had a size of 35 animals in early summer 2022. A total of five animals had died peracutely within a few weeks before the visit in August 2022. In addition, two crias had been born during the same period, resulting in a total of 32 animals (23 llamas and nine alpacas) at the time of our visit. The age of the animals ranged from 10 days to 19 years, all animals had been shorn between April and May 2022. The purpose of the visit was to check the health status of the remaining animals in the herd after the previously incurred losses. A clinical examination of each of the animals according to the routine protocol of the clinic was performed and the animals were vaccinated against clostridia. The BCS of the animals as part of the clinical examination was assessed by six examiners in order to increase the precision of the results and to obtain more routine in herd management of SAC. The assessment of the BCS is seen as a routine method in SAC husbandry, which should acclimatize the animals to stress-free handling (26). Since not all examiners assessed the BCS in three of the animals, these animals were excluded from the evaluation. Ultimately, body condition scores of 20 llamas and nine alpacas assessed by six examiners were included in this study.

2.2. Assessment of BCS

The BCS was assessed by palpation of the lumbar spine behind the last ribs according to previous descriptions (6, 9, 10) and ranged from 1 to 5 as follows:

BCS 1—emaciated
BCS 2—thin
BCS 3—optimal
BCS 4—overweight
BCS 5—obese

All examiners palpated the spinous and transverse processes of the lumbar vertebrae as well as the muscle and fat coverage in between. In animals with an optimal nutritional status (BCS 3), the line between spinous and transverse processes should be neither convex nor concave (Figure 1). The more concave the line was, the lower the BCS was classified (BCS 1 and 2), the more convex the line was, the higher the BCS was classified (BCS 4 and 5). Steps of 0.5 in between were possible. Most of the animals were fixed in a chute for clinical examination. A few animals that were not compatible with the chute were restrained by only one person for the examination.

The results of the individual examiners for each animal were recorded as paper protocols on the farm and transferred to an Excel sheet (Microsoft Excel for Office 365) for further analysis later.

2.3. Examiners

The six different examiners had different levels of experience with body condition scoring in SAC:

- Examiner 1: veterinarian with more than 5 years of experience of regular practical assessment of BCS in SAC and small ruminants at clinic and herd level prior to the study
- Examiner 2: veterinarian with approx. one year of experience in regular practical assessment of BCS in SAC and small ruminants at clinic level prior to the study
- Examiner 3: veterinarian with approx. one year of experience in regular practical assessment of BCS in SAC and small ruminants at clinic level prior to the study
- Examiner 4: veterinarian with approx. one year of experience in regular practical assessment of BCS in small ruminants at herd level prior to the study
- Examiner 5: veterinary student who had learnt to assess BCS in SAC 3 years prior to the study
- Examiner 6: animal keeper, owner of the farm with more than 5 years of experience in regular practical assessment of BCS in SAC at herd level prior to the study.

2.4. Gold Standard BCS (gsBCS)

In order to obtain a "correct" BCS as a reference value for each animal, a gold standard BCS (gsBCS) was calculated for each animal according to Kleiböhmer et al. (19) who checked the accuracy of the BCS in cattle (19). Due to the experience and the close agreement of examiners 1 and 6, the gsBCS was calculated from their findings by calculating the means of both examiners for each animal. Examiners 1 and 6 both had more than 5 years of experience in determining the BCS. Examiner 6 tended to assess a lower BCS than examiner 1. In 16 animals, examiners 1 and 6 agreed, in seven animals, the BCS assessed by examiner 6 was 0.5 score points lower than that assessed by examiner 1 and in two animals, 1 score point lower than examiner 1. In four animals, examiner 6 was 0.5 score points higher

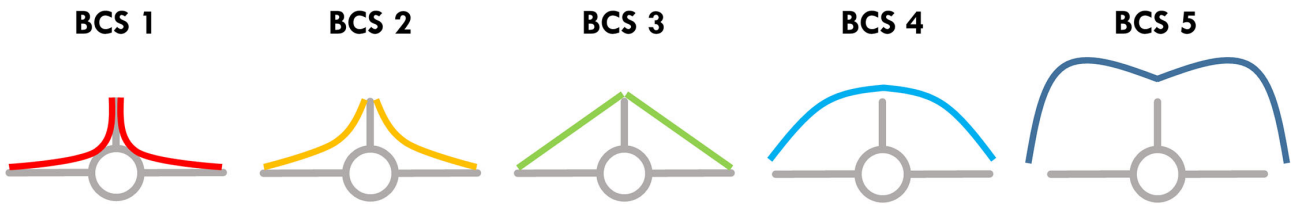

FIGURE 1
Schematic cross section through the lumbar spine. BCS in llamas and alpacas is assessed by palpating the tissue coverage of the lumbar vertebrae. The spinous and transverse processes as well as the connecting line between these two are palpated. If the BCS is optimal (3), this connecting line is straight; if this line is concave, the BCS is <3; if it is convex, the BCS is >3. Figure modified according to Wagener and Ganter (9).

than examiner 1. Since the two examiners differed by 0.5 score points for 11 animals, the calculated gsBCS for these animals resulted in 0.25 score points. Although these were mathematically correct, they did not represent a BCS that could be realistically examined. Therefore, for these animals, the BCS was rounded up or down to the nearest full score. For example, if the calculated value was 2.25, it was rounded down to 2, and if it was 2.75, it was rounded up to 3.

2.5. Statistical evaluation

Analysis of data was performed with Excel (Microsoft Excel for Office 365), SAS (SAS Enterprise Guide 7.1) and R [(R Foundation for Statistical Computing, Vienna, Austria, https://www.R-project.org) in combination with RStudio (Integrated Development for RStudio, Inc., http://www.rstudio.com)].

Descriptive statistics included mean, minimum and maximum of the BCS in each animal as well as mean, minimum and maximum of the assessed BCS by each examiner. In some of the groups examined, the values were not normally distributed. However, the mean was consistently used in the descriptive statistics, since some gradations were not visible in the median. In addition, the number of deviations from gsBCS were determined for each examiner by subtraction. For testing the inter-rater reliability of a BCS in ruminants, different statistical tests have been used in previously published studies (21, 22, 24, 27, 28). We used Spearman's rank correlation coefficient (r), Kendall's rank correlation coefficient (τ), and Cohen's weighted kappa (κ_w) for testing pairwise correlation and agreement of the examiners with each other and with the gsBCS. In addition, one overall Kendall's coefficient of concordance (W) was computed, including only the examiners' scores without the gsBCS. Spearman's and Kendall's correlation was interpreted as follows: r/τ = 0–0.1: negligible correlation; r/τ = 0.1–0.39: weak correlation; r/τ = 0.4–0.69: moderate correlation; r/τ = 0.7–0.89: strong correlation; r/τ = 0.9–1.0: very strong correlation (29, 30). Cohen's weighted kappa and Kendall's coefficient of concordance were interpreted as follows: κ_w/W = 0–0.2: slight agreement; κ_w/W = 0.21–0.4: fair agreement; κ_w/W = 0.41–0.6: moderate agreement; κ_w/W = 0.61–0.8: substantial agreement; κ_w/W = 0.81–1: almost perfect (31). Differences between llamas and alpacas were tested by using the unpaired two-samples Wilcoxon test. A p-value <0.05 was considered significant.

3. Results

By the examination of 29 animals, in total, 174 BCSs were recorded. All assessed BCSs as well as further details on the animals can be found in Table 1. The mean value for all records was 3.29, the lowest BCS was 1.5, the highest 5. The gsBCS for all animals was 3.28 (mean) and ranged from 1.5 to 4. The alpacas of this herd had a lower gsBCS (mean: 2.83) than the llamas (mean: 3.48). However, the difference was not statistically significant ($p = 0.08$). The minimal BCS assessed by an examiner for all animals was 2.83 (mean) with a range of 1.5–4; the maximal BCS assessed by an examiner for all animals was 3.74 (mean) with a range of 2–5. The range of the BCSs that were assessed in an individual animal by the six examiners was 0.91 (mean) for all animals and was between 0 and 2 score points. In only one animal with a BCS of 4 did all six examiners give the same BCS. In 11 animals, the range of the examiners was 0.5 score points, the mean gsBCS in these animals was 3.00. In another 11 animals with a mean gsBCS of 3.18, the range was one score point. Four animals with a gsBCS of 3.75 (mean) had a range of 1.5 score points and two animals with a gsBCS of 4 each had a range of 2 score points in the BCS assessed by the six examiners.

Deviations of the individual examiners are displayed in Table 2. For five of the six examiners no significant difference could be detected between the examination of the BCS in alpacas and lamas regarding the deviations in the assessed BCS from the gsBCS (examiner 1: $p = 0.38$; examiner 2: $p = 0.03$; examiner 3: $p = 0.21$; examiner 4: $p = 0.96$; examiner 5: $p = 0.74$; examiner 6: $p = 0.67$).

Spearman's correlation analysis revealed strong significant correlations between gsBCS and examiners 2, 4, and 5 and moderate significant correlations between gsBCS and examiner 3. Interpreting the correlation and agreement between gsBCS and examiners 1 and 6 is unnecessary, since the gsBCS is the result of the assessments by examiners 1 and 6. Spearman's correlations between the individual examiners were almost all strong, moderate correlations were only found between examiner 3 and other examiners (1,2,5). The range for r between the individual examiners was 0.52–0.89.

When the same limits were applied to τ, Kendall's rank correlation coefficient resulted in weaker correlations. In this statistic, examiner 4 showed a strong correlation with the gsBCS and examiners 2, 3, and 5 a moderate correlation therewith. There was a moderate correlation among the individual examiners. A strong correlation was only found between examiner 1 and examiners 4, 5, and 6 as well as between examiner 4 and examiners 5 and 6. The range for τ between the individual examiners was 0.43–0.80.

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TABLE 1 Overview of sex, age, assessed Body Condition Score (BCS) by each examiner, and calculated gsBCS (gold standard BCS) of the examined alpacas and llamas.

Animal	Species	Sex*	Age (years)	Examiner 1	Examiner 2	Examiner 3	Examiner 4	Examiner 5	Examiner 6	gsBCS
A 1	Alpaca	m	0	4	4	3.5	3.5	4	3.5	4
A 2	Alpaca	f	1	4	4	2.5	4.5	4	4	4
A 3	Alpaca	f	1	3.5	3.5	3	3.5	3.5	3.5	3.5
A 4	Alpaca	f	2	2.5	3	2.5	2.5	2.5	3	3
A 5	Alpaca	f	5	3.5	3.5	3.5	3	3	3.5	3.5
A 6	Alpaca	f	7	2.5	3	2.5	2.5	2.5	2	2
A 7	Alpaca	f	10	2	2	3	2.5	2.5	2	2
A 8	Alpaca	f	13	1.5	1.5	2	1.5	1.5	1.5	1.5
A 9	Alpaca	mn	13	2	2.5	2	2	2.5	2.5	2
L 1	Llama	m	1	3	3.5	3	3	2.5	3	3
L 2	Llama	f	2	3	4	4.5	3.5	3	3	3
L 3	Llama	f	2	3.5	3.5	3.5	4	4	3.5	3.5
L 4	Llama	f	3	3.5	3.5	4	4	3.5	4	4
L 5	Llama	m	4	3.5	3	4	3.5	3.5	3.5	3.5
L 6	Llama	f	5	3.5	4	3	4	3.5	3.5	3.5
L 7	Llama	mn	5	3.5	3.5	4	5	3	4	4
L 8	Llama	f	6	4.5	4.5	5	5	3.5	4	4
L 9	Llama	f	6	3	4	4	4	3.5	4	3.5
L 10	Llama	f	7	4	4	4	4.5	3.5	4	4
L 11	Llama	f	8	3	3	2.5	3	2.5	3	3
L 12	Llama	m	9	4	4	4	4	4	4	4
L 13	Llama	f	10	3.5	3	4	3.5	3.5	3.5	3.5
L 14	Llama	f	11	2.5	2	2.5	2.5	2.5	2.5	2.5
L 15	Llama	m	11	4.5	3.5	4	4	3.5	4	4
L 16	Llama	mn	14	3.5	3.5	4	3.5	2.5	4	4
L 17	Llama	f	14	3.5	3.5	4	5	4	4.5	4
L 18	Llama	mn	16	2.5	3	3	3	3	2.5	2.5
L 19	Llama	f	19	2	2	3	2	2	2.5	2
L 20	Llama	mn	19	3.5	3.5	3.5	3.5	3	4	4

*f, female; m, male; mn, male neutered.

Cohen's weighted kappa (κ_w), on the other hand, showed better agreement than τ in most comparisons. The gsBCS had a substantial agreement with examiners 2–5. The kappa between examiners showed substantial agreement in almost all pairs except in the comparison of examiner 2 with examiners 3 and 5, and examiner 5 with examiners 3 and 4. The range for κ_w between the individual examiners was 0.50–0.79.

Kendall's coefficient of concordance (W) amounted to 0.78, which corresponded to an overall substantial agreement between the six examiners.

The exact values for Spearman's rank correlation coefficient, Kendall's rank correlation coefficient, and Cohen's weighted kappa are displayed in Tables 3, 4.

4. Discussion and outlook

When considering the overall Kendall's coefficient of concordance, the agreement between the estimated BCSs of different examiners in this mixed herd of llamas and alpacas was surprisingly high. The largest range in BCS with 2 score points was found in only two of the animals, whereas the assessed BCS in 22 of the 29 animals (75.9%) differed only by a maximum of 1

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TABLE 2 Overview of the results of each examiner and the number of assessed Body Condition Scores (BCS) that deviated from the gsBCS (gold standard BCS).

					Deviation from gsBCS			
	n	Mean	Min-Max	Total score	± 0.00 BCS	± 0.50 BCS	± 1.00 BCS	± 1.50 BCS
All examiners	174	3.29	1.5–5	573	92 (52.9%)	69 (39.7%)	10 (5.7%)	3 (1.7%)
gsBCS	29	3.28	1.5–4	95	0	0	0	0
Examiner 1	29	3.21	1.5–4.5	93	19 (65.5%)	10 (34.5%)	0 (0.0%)	0 (0.0%)
Examiner 2	29	3.29	1.5–4.5	95.5	12 (41.4%)	15 (51.7%)	2 (6.9%)	0 (0.0%)
Examiner 3	29	3.38	2–5	98	12 (41.4%)	12 (41.4%)	3 (10.3%)	2 (6.9%)
Examiner 4	29	3.45	1.5–5	100	12 (41.4%)	14 (48.3%)	3 (10.3%)	0 (0.0%)
Examiner 5	29	3.10	1.5–4	90	13 (44.8%)	13 (44.8%)	2 (6.9%)	1 (3.4%)
Examiner 6	29	3.33	1.5–4.5	96.5	24 (82.8%)	5 (17.2%)	0 (0.0%)	0 (0.0%)

TABLE 3 Spearman's rank correlation coefficient (r), Kendall's rank correlation coefficient (τ), and Cohen's weighted kappa (κ_w) are listed in bold.

Spearman's rank correlation coefficient (r) Kendall's rank correlation coefficient (τ) Cohen's weighted kappa (κ_w)							
	gsBCS	Examiner 1	Examiner 2	Examiner 3	Examiner 4	Examiner 5	Examiner 6
gsBCS		*<0.0001* *<0.0001* –	*<0.0001* *<0.0001* –	*<0.0001* *0.0002* –	*<0.0001* *<0.0001* –	*<0.0001* *<0.0001* –	*<0.0001* *<0.0001* –
Examiner 1	**0.91** **0.86** **0.86**		*<0.0001* *<0.0001* –	*0.0003* *0.0007* –	*<0.0001* *<0.0001* –	*<0.0001* *<0.0001* –	*<0.0001* *<0.0001* –
Examiner 2	**0.73** **0.65** **0.73**	**0.77** **0.69** **0.78**		*0.0007* *0.0009* –	*<0.0001* *<0.0001* –	*<0.0001* *<0.0001* –	*<0.0001* *<0.0001* –
Examiner 3	**0.66** **0.57** **0.66**	**0.62** **0.52** **0.64**	**0.59** **0.51** **0.58**		*<0.0001* *<0.0001* –	*0.0035* *0.0054* –	*<0.0001* *<0.0001* –
Examiner 4	**0.86** **0.78** **0.63**	**0.82** **0.73** **0.74**	**0.79** **0.69** **0.69**	**0.70** **0.61** **0.69**		*<0.0001* *<0.0001* –	*<0.0001* *<0.0001* –
Examiner 5	**0.73** **0.64** **0.71**	**0.80** **0.70** **0.74**	**0.70** **0.61** **0.50**	**0.52** **0.43** **0.54**	**0.81** **0.71** **0.54**		*<0.0001* *<0.0001* –
Examiner 6	**0.95** **0.92** **0.92**	**0.83** **0.74** **0.79**	**0.72** **0.62** **0.71**	**0.70** **0.60** **0.67**	**0.89** **0.80** **0.75**	**0.70** **0.60** **0.67**	

The first line in each cell represents Spearman's r, the second line Kendall's τ, and the third line Cohen's κ_w. Respective *p*-values for Spearman's r and Kendall's τ are listed in italics. gsBCS, gold standard BCS.

score point between the examiners. When using the gsBCS as a definition of the correct score in each animal, only three (1.7%) of the 174 BCSs that were assessed in this study deviated from the gsBCS by more than 1 score point. Of course, this has to be considered within the context of the limitation that the gsBCS was calculated from the findings of examiners 1 and 6, and thus, there was already a close relationship between these three values. When interpreting the deviations from the gsBCS, the other examiners (2–5) did not deviate from the gsBCS in more than 40% of the animals, and did not deviate more than 0.5 score points in over 80% of the animals.

Examiners 1, 4, and 6 had the highest means for r, τ and κ_w compared to the other examiners. In contrast to the others, these examiners had more experience in assessing BCS in flocks. Furthermore, examiners 1 and 6 had the longest experience in assessing BCS in SAC. The other examiners who had clinical but not flock experience in assessing the BCS in SAC resulted in lower means for r, τ and κ_w. In contrast to the SACs detected in the clinic, the SACs

TABLE 4 Agreements [Spearman's rank correlation coefficient (r), Kendall's rank correlation coefficient (τ) and Cohen's weighted kappa (κ_w)] of all examiners with the gsBCS (line "gsBCS"; $n = 6$), and of the individual examiners with each of the other examiners (lines "examiner 1" to "examiner 6"; $n = 5$ each).

	$r =$		$\tau =$		$\kappa_W =$	
	Mean±SD	Min–Max	Mean±SD	Min–Max	Mean±SD	Min–max
gsBCS	0.81 ± 0.12	0.66–0.95	0.74 ± 0.14	0.57–0.92	0.75 ± 0.11	0.63–0.92
Examiner 1	0.77 ± 0.09	0.62–0.83	0.68 ± 0.09	0.52–0.75	0.74 ± 0.06	0.64–0.79
Examiner 2	0.71 ± 0.08	0.59–0.79	0.62 ± 0.07	0.51–0.69	0.65 ± 0.11	0.50–0.78
Examiner 3	0.63 ± 0.08	0.52–0.70	0.53 ± 0.07	0.43–0.61	0.62 ± 0.06	0.54–0.69
Examiner 4	0.80 ± 0.07	0.70–0.89	0.71 ± 0.07	0.61–0.80	0.68 ± 0.08	0.54–0.74
Examiner 5	0.71 ± 0.12	0.52–0.81	0.61 ± 0.11	0.43–0.71	0.60 ± 0.10	0.50–0.74
Examiner 6	0.77 ± 0.09	0.70–0.89	0.67 ± 0.09	0.60–0.80	0.72 ± 0.05	0.67–0.79

examined in this study had higher BCSs. The alpacas referred to our clinic revealed a BCS of 2.43 ± 0.77 (mean ± SD), the llamas a BCS of 2.20 ± 0.99 (mean ± SD) (5). This may have resulted in lower BCS being detected more reliably, and could be an explanation as to why animals with a higher gsBCS revealed a higher range of assessed BCS by the individual examiners. It is worth mentioning that the greatest differences in the estimation of the BCS between the examiners were in animals with a mean gsBCS of around 3, that represents an optimal nutritional status. The clinical consequences of these differences are therefore negligible.

Since no comparable studies for SAC are known so far, the results of studies in cattle and sheep were used for comparison. Kleiböhmer et al. (19) found that even inexperienced examiners who had received extensive training in BCS assessment were able to obtain reproducible BCS assessment results after 6 weeks (19). The 175 cows in their study were examined by 15 examiners. Herein, only 3% of the assessed BCS had a deviation of 0.5 score points from the gsBCS.

Other studies on the inter-rater reliability used a weighted kappa analysis for evaluation (21, 22, 24). In our study, the range of inter-rater reliability among examiners was $\kappa_w = 0.50–0.79$, which is comparable to other studies on the inter-rater reliability of the BCS in sheep or cattle.

Phythian et al. (22) investigated the inter-rater reliability for body condition scoring in sheep before and after a brief recalibration on the inter-observer agreement of three examiners (22). Before re-calibration, they found $\kappa_w = 0.3–0.5$ and $W = 0.4–0.5$, and thereafter, $\kappa_w = 0.4–0.7$ and $W = 0.4–0.6$. They also concluded that both a BCS as well in full as in half-unit scores can be determined by different examiners with a good agreement. In a study from New Zealand by Corner-Thomas et al. (24), BCSs of 45 sheep were assessed by both three experienced technicians and 23 farmers who had previously received training in BCS. Pairs of farmers revealed a higher variability in kappa ($\kappa_w = 0.54–0.94$) than the pairs of technicians ($\kappa_w = 0.82–0.88$) (24).

Kristensen et al. (21) tested the inter-rater reliability of 51 dairy veterinarians with different levels of experience after a workshop on BCS (21). The examiners assessed the BCS of 20 cows twice at an interval of 2.5 hours. The inter-rater reliability between the workshop participants was tested as well as the inter-rater reliability between participants and the six instructors who had also received a special training beforehand. That study showed that the inter-rater reliability of the second scoring showed better agreements (κ_w scoring 1 between workshop participants: $\kappa_w = 0.50/0.17/0.78$ [mean/minimum/maximum] κ_w scoring 2 between workshop participants: $\kappa_w = 0.64/0.41/0.82$). In addition, the respective pairs of workshop participants and instructors revealed a higher agreement than between workshop participants (κ_w scoring 1 between workshop participants and instructors: $\kappa_w = 0.62/0.33/0.84$ [mean/minimum/maximum]; κ_w scoring 2 between workshop participants: $\kappa_w = 0.74/0.55/0.85$) (21). This is also consistent with the findings from our study: examiners 1 and 6, who both had the longest experience in body condition scoring at SAC and could thus be compared with the instructors from the study by Kristensen et al. (21), had the highest kappa values compared to the other examiners.

However, when comparing the BCS in SAC to the BCS in cows, it is important to note that the BCS in cows involves multiple body regions, which enables a more precise awarding of 0.25 score points (18). In our study, where BCS was only assessed by palpation of the lumbar spine, such a precision cannot be achieved under practical conditions (9). The comparison to previous studies in sheep (22, 24), where the BCS was assessed in a similar manner, therefore seems more apt. The influence of different examiners concerning other body regions needs to be studied separately. This is supported by the findings of Zielke et al. (28) who found differences in the inter-rater reliability of BCS assessed in different body regions in bisons (28).

Since only inter-rater reliability of the BCS in SAC was evaluated in our study, intra-rater reliability has so far not been taken into account. The latter describes how reproducible the assessment of the BCS in an animal by the same examiner is. Intra-rater reliability of the BCS in cows and sheep has been studied by different research groups so far (20–24, 27, 32). Data on intra-rater reliability from ruminants suggest that more experienced examiners achieve higher kappa values than less experienced examiners (20, 21). This still remains to be tested for SAC. Kristensen et al. (21) also concluded that even limited training can lead to a significant improvement in validity and precision in the assessment of BCS (21).

Approaches for BCS assessment are not only available for the New World camelids but also for the Old World camelids in which different regions of the body, including the hump, are included (33–36). To date, there have been no studies on how reproducible the results are for assessing BCS in Old World camelids. Since the BCS could also provide an important indication of nutritional status and possible infections with gastrointestinal endoparasites in both New and Old World camels, the accuracy and repeatability of the BCS should also be investigated more closely in these species.

In conclusion, our findings indicate that the assessment of the BCS at the lumbar spine in SAC is a quite reproducible examination method, even when it is performed by different examiners. Our data as well as the results from other studies support the assumption that reproducibility increases with training and experience. If BCS is assessed regularly by staff involved in husbandry and veterinary care of SAC, emaciation as a sign of disease, stress, or lack of management can be detected at an early stage and appropriate measures of intervention can be taken in time.

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.

Ethics statement

Ethical review and approval were not required for the animal study because all data used for this study were collected during the clinical examination of the animals for diagnosis of a veterinary herd problem. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MGW wrote the manuscript, the manuscript was reviewed by JS, NO, AT, FK, and MG, data were collected by MGW, JS, NO, AT, and FK, and data were evaluated by FK and MGW. The study was designed by MGW and supervised by MG and FK. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Molecular detection and characterization of *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bieneusi* infections in dromedary camels (*Camelus dromedaries*) in Egypt

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Introduction: Few studies have investigated the occurrence of microeukaryotic gut parasites in dromedary camels in Egypt, and the majority of these investigations are based on microscopic analysis of fecal material.

Methods: Herein, we assessed the occurrence, molecular diversity, and zoonotic potential of protozoan (*Cryptosporidium* spp. and *Giardia duodenalis*) and microsporidian (*Enterocytozoon bieneusi*) pathogens in individual fecal samples (n = 102) of dromedary camels with (n = 26) and without (n = 76) diarrhea from Aswan Governorate, Upper Egypt. Other factors possibly associated with an increased risk of infection (geographical origin, sex, age, and physical condition) were also analyzed. The *SSU* rRNA or ITS genes were targeted by molecular (PCR and Sanger sequencing) techniques for pathogen detection and species identification.

Results and discussion: The most abundant species detected was *G. duodenalis* (3.9%, 4/102; 95% CI: 1.1–9.7), followed by *Cryptosporidium spp*. (2.9%, 3/102; 95% CI: 0.6–8.4). All samples tested negative for the presence of *E. bieneusi*. Sequence analysis data confirmed the presence of zoonotic *C. parvum* (66.7%, 2/3) and cattle-adapted *C. bovis* (33.3%, 1/3). These *Cryptosporidium* isolates, as well as the four *Giardia*-positive isolates, were unable to be amplified at adequate genotyping markers (*Cryptosporidium*: *gp60*; *Giardia*: *gdh*, *bg*, and *tpi*). Camels younger than 2 years old were significantly more likely to harbor *Cryptosporidium* infections. This connection was not statistically significant, although two of the three cryptosporidiosis cases were detected in camels with diarrhea. The spread of *G. duodenalis* infections was unaffected by any risk variables studied. This is the first report of *C. parvum* and *C. bovis* in Egyptian camels.

The finding of zoonotic *C. parvum* has public health implications since camels may function as sources of oocyst pollution in the environment and potentially infect livestock and humans. Although preliminary, this study provides useful baseline data on the epidemiology of diarrhea-causing microeukaryotic parasites in Egypt. Further research is required to confirm and expand our findings in other animal populations and geographical regions of the country.

KEYWORDS

epidemiology, genotyping, protists, microsporidia, Zoonoses, transmission

1. Introduction

Globally, *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bieneusi* are among the most prevalent diarrhea-causing enteric parasites in humans and livestock (1–5). These pathogens cause significant morbidity and, in the case of *Cryptosporidium*, mortality in children <5 years old and immunocompromised persons residing in low-resource settings with little or no access to safe drinking water and sanitation facilities (6, 7). They also pose a threat to public health in middle- and high-income nations (8). These pathogens are transmitted through the fecal–oral route or by direct contact with infected animals or humans. Adult livestock infected with *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi* are usually asymptomatic carriers that release varied amounts of (oo)cysts/spores into the surrounding environment and remain a potential source of infection for other animals and humans (9, 10). However, infected neonatal animals may have diarrhea, loss of appetite, lethargy, dehydration, and in some cases, death can occur (11, 12). Importantly, infected neonatal animals can release substantial quantities of instantly infectious (oo)cysts/spores (13, 14), making them important contributors to the (oo)cysts/spore burden in the environment, including surface waters meant for human consumption (15).

Many clinical research facilities in low-income countries rely on microscopy analyses of fecal smears to diagnose enteric parasites (16). Although this method is cheap and easy to perform, it requires well-trained and experienced microscopists, takes time, and lacks diagnostic sensitivity (17). To overcome these limitations, several molecular biological methods for detecting and distinguishing microeukaryotic intestinal parasites have been developed. These include PCR-based genotyping, Sanger sequencing of PCR products, and fluorescence probe-based qPCR techniques (18–20). Molecular methods to improve epidemiological and epidemic studies by allowing researchers to monitor pathogen infection sites, transmission pathways, and virulent genetic variants. For this task, highly sensitive, multi-copy genes, including the small subunit ribosomal RNA (*SSU* rRNA) and the ribosomal internal transcribed spacer (ITS) markers, are widely used (21).

At least 44 *Cryptosporidium* species are considered taxonomically valid (22, 23). Nearly 15 species (*C. andersoni*, *C. bovis*, *C. erinacei*, *C. felis*, *C. hominis*, *C. macropodum*, *C. muris*, *C. occultus*, *C. parvum*, *C. ryanae*, *C. scrofarum*, *C. suis*, *C. tyzzeri*, *C. ubiquitum*, and *C. xiaoi*) have been reported in domestic ruminants globally, with *C. parvum* the most dominant species, particularly in cattle (3, 20, 24). Seven *Cryptosporidium* species (*C. andersoni*, *C. bovis*, *C. hominis*, *C. muris*, *C. occultus*, *C. parvum*, and *C. ubiquitum*), and two genotypes (rat IV and camel) have been identified circulating in camels to date (Table 1).

Giardia duodenalis (syn. *G. intestinalis* and *G. lamblia*) is the only *Giardia* species able to infect domestic ruminants (22, 76). *Giardia duodenalis* is considered a complex cryptic species with eight distinct genetic variants (assemblages A to H), which differ in host distribution and specificity. Assemblages A and B are found in humans and in many other mammals, whereas C and D are found in canids, E in wild and domestic ungulates, F in felids, G in rodents, and H in marine pinnipeds (22, 76). Camels seem to be primarily infected by ungulate-adapted *G. duodenalis* assemblage E; however, zoonotic assemblage A infections have also been reported (Table 1). Remarkably, assemblage E is responsible for 8–100% of cases of human giardiasis documented in Egypt (77–79). More than 600 *E. bieneusi* genotypes have been identified and classified into 11 major phylogenetic groups, of which groups 1 and 2 contain most genotypes with zoonotic potential, and the remaining groups 3–11 include largely host-adapted genotypes associated with specific animal species (80, 81). Today, 15 *E. bieneusi* genotypes have been identified in camels globally, with CAM1 and EbpC accounting for nearly 70% of infections detected (Table 1).

Dromedary camels (*Camelus dromedaries*) have a significant economic, social, and ecological role in nomadic and/or pastoralist communities living in arid or semi-arid regions globally (79). They are natural hosts for a wide range of protists (*Balantioides coli*, *Blastocystis* sp., *Cryptosporidium* spp., *Enterocytozoon bieneusi*, *Giardia* spp., *Toxoplasma gondii*, and *Trypanosoma* spp.), helminth (*Echinococcus granulosus*, *Fasciola hepatica*, *Schistosoma* spp., and *Trichinella spiralis*), and arthropod (*Linguatula serrata* and *Sarcoptes scabiei*) zoonotic species, representing an often-unrecognized public health threat (27, 82, 83). In addition, infections by some of these pathogens result in significant economic loss due to decreased milk and meat output, diminished fertility, and mortality (84–86).

Several studies in Egypt have looked at the presence of parasite infections, such as *Anaplasma*, *Babesia*, *Echinococcus*, *Sarcocystis*, *Sarcoptes*, *Theileria*, and *Trypanosoma* in dromedary camels (87–90). However, evidence on the presence of *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi* is even scarcer, with the drawback that most available data come from outdated microscopy-based studies (Table 1). Previous studies have suggested that camels infected with

TABLE 1 Global occurrence and genetic diversity of *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bieneusi* reported in camelids including Bactrian (*Camelus bactrianus*) and dromedary (*Camelus dromedaries*) camels.

Pathogen	Host	Country	Detection method	Frequency % (no. pos./total)	Species identified (no.)	Genotype (no.)	References
Cryptosporidium spp.	DC	Algeria	CM, PCR	5.1 (2/39)	*C. parvum* (2)	If-like (2)	(25)
	DC	Algeria	CM	2.0 (3/149)	*Cryptosporidium* spp. (3)	–	(26)
	DC	Algeria	CM	1.8 (13/717)	*Cryptosporidium* spp. (13)	–	(27)
	DC	Algeria	CM	58.0 (58/100)	*Cryptosporidium* spp. (58)	–	(28)
	DC	Algeria	CM, PCR	10.0 (4/40)	*Cryptosporidium* spp. (4)	ND	(29)
	DC	Australia	PCR	–[a] (1/1)	*C. parvum* (1)	IIaA17G2R1	(30)
	DC	Azerbaijan	CM	35.7 (65/182)	*C. andersoni*[b] (NA), *C. muris*[b] (NA)	–	(31)
	DC	China	PCR-RFLP	50.0 (2/4)	*C. andersoni* (2)	–	(32)
	DC	Egypt	CM	3.7 (37/1,097)	*Cryptosporidium* spp. (37)	–	(33)
	DC	Egypt	CM	17.5 (14/80)	*Cryptosporidium* spp. (14)	–	(34)
	DC	Egypt	CM	3.8 (4/101)	*Cryptosporidium* spp. (4)	ND	(35)
	DC	Egypt	CM, PCR	19.4 (28/145)	*C. muris* (NA)	–	(36)
	DC	Egypt	CM	24.2 (29/120)	*Cryptosporidium* spp. (29)	–	(37)
	DC	Egypt	PCR-RFLP	5.9 (6/101)	*C. parvum* (2), rat genotype IV (1), and camel genotype (3)	IIaA15G1R1 (1), IIdA19G1 (1)	(38)
	DC	Egypt	CM	8.3 (10/120)	*Cryptosporidium* spp. (10)	–	(39)
	DC	Egypt	CM	20.0 (50/248)	*Cryptosporidium* spp. (50)	–	(40)
	DC	Ethiopia	CM	25.1 (77/307)	*Cryptosporidium* spp. (77)	–	(41)
	DC	Iran	CM	3.3 (13/396)	*Cryptosporidium* spp. (13)	–	(42)
	DC	Iran	CM	1.9 (6/306)	*Cryptosporidium* spp. (6)	–	(43)
	DC	Iran	CM, ELISA	37.9 (39/103)	*Cryptosporidium* spp. (39)	–	(44)
	DC	Iran	CM, ELISA	16.9 (11/65)	*Cryptosporidium* spp. (11)	–	(45)
	DC	Iran	CM, ELISA	4.7 (4/85)	*C. andersoni* (1), *C. muris* (1), and *C. parvum* (2)	–	(46)
	DC	Iran	CM	20.3 (61/300)	*Cryptosporidium* spp. (61)	–	(47)
	DC	Iran	CM	10.0 (17/170)	*Cryptosporidium* spp. (17)	–	(48)
	DC, BC	Iran	CM	81.8 (36/44)	*Cryptosporidium* spp. (36)	–	(49)
	DC	Iran	ELISA	0.5 (1/184)	*C. parvum* (1)	–	(50)
	DC	Iraq	CM	61.0 (61/100)	*Cryptosporidium* spp. (61)	–	(51)
	DC	Iraq	PCR	14.0 (7/50)	*C. parvum* (7)	ND	(52)
	DC	Iraq	CM	55.0 (110/200)	*Cryptosporidium* spp. (110)	–	(53)
	DC	Iraq	CM	37.5 (45/120)	*Cryptosporidium* spp. (45)	–	(54)
	DC	Kuwait	CM	4.0 (10/253)	*Cryptosporidium* spp. (10)	–	(55)
	DC	Saudi Arabia	CM, ELISA	18.4 (9/49); 22.4 (11/49)	*Cryptosporidium* spp. (9–11)	–	(56)
	DC	Saudi Arabia	CM	15.1 (6/33)	*Cryptosporidium* spp. (6)	–	(57)
	DC	Saudi Arabia	ELISA	17.4 (16/92)	*C. parvum* (16)	–	(58)
	BC	China	PCR	–[a] (1/1)	*C. andersoni* (1)	–	(59)
	BC	China	PCR	–[a] (1/2)	*C. andersoni* (1)	–	(60)
	NA	China	PCR	15.0 (6/40)	*C. andersoni* (4), *C. bovis* (2)	ND	(61)
	BC	China	PCR	7.6 (36/476)	*C. andersoni* (24), *C. bovis* (1), *C. hominis* (1), *C. occultus* (2), *C. parvum* (6), and *C. ubiquitum* (2)	If-like (5), IkA19G1 (1), IIdA15G1 (1), and XIIa (2)	(62)

(Continued)

TABLE 1 (Continued)

Pathogen	Host	Country	Detection method	Frequency % (no. pos./total)	Species identified (no.)	Genotype (no.)	References
	BC	China	PCR	–[a] (2/2)	*C. muris* (2)	–	(63)
	BC	China	PCR	15.0 (6/40)	*Cryptosporidium* spp. (6)	ND	(64)
	BC	China	PCR	NA	*C. muris* (4)	–	(65)
	BC	Czech Republic	PCR	–[a] (2/2)	*C. muris* (2)	–	(66)
	BC	Czech Republic	PCR	–[a] (2/2)	*C. andersoni* (2)	–	(67)
	BC	Czech Republic	PCR	–[a] (1/1)	*C. muris* (1)	–	(68)
	BC	USA	CM	–[a] (1/1)	*Cryptosporidium* spp. (1)	ND	(69)
	BC	USA	PCR	–[a] (1/1)	*C. muris* (1)	ND	(70)
	BC, DC	USA	CM	1.3 (1/77)	*Cryptosporidium* spp. (1)	–	(71)
Giardia duodenalis	DC	Egypt	CM	5.0 (6/120)	*G. duodenalis* (6)	–	(37)
	DC	Iraq	CM	–[a] (4/4)	*G. duodenalis* (4)	–	(72)
	DC	Iraq	CM	**24.0 (24**/100)	*G. duodenalis* (24)	–	(51)
	DC	Iraq	CM	20.0 (40/200)	*G. duodenalis* (40)	ND	(53)
	DC	Iraq	CM	4.2 (5/120)	*G. duodenalis* (5)	–	(54)
	DC	Saudi Arabia	CM	–[a] (7/7)	*G. duodenalis* (7)	–	(73)
	DC, BC	USA	CM	1.3 (1/77)	*G. duodenalis* (1)	–	(71)
	BC	China	PCR	7.5 (3/40)	*G. duodenalis* (3)	A (1), E (2)	(61)
	BC	China	PCR	9.8 (84/852)	*G. duodenalis* (84)	A (14), E (23). A+E (1)	(74)
	BC	China	PCR	7.5 (3/40)	*G. duodenalis* (3)	ND	(64)
	BC	China	PCR	NA	*G. duodenalis* (NA)	A (1) and E (1)	(65)
Enterocytozoon bieneusi	DC	Algeria	PCR	20.5 (8/39)	*E. bieneusi* (8)	Camel-2 (2) and Macaque1 (6)	(25)
	BC	China	PCR	30.0 (122/407)	*E. bieneusi* (122)	BEB6 (1), CAM1 (72), CAM2 (8), CAM3 (1), CAM4 (5), CAM5 (1), CAM6 (1), CHG16[c] (1), CM8 (1), EbpA (5), EbpC (23), Henan-IV (1), O (1), and WL17[d] (1)	(75)
	BC	China	PCR	45.0 (18/40)	*E. bieneusi* (18)	BEB6 (3), CAM1 (8), and CAM2 (7)	(61)
	BC	China	PCR	NA	*E. bieneusi* (NA)	CD7 (3) and CHS9 (1)	(65)
	BC	China	PCR	45.0 (18/40)	*E. bieneusi* (18)	ND	(64)

ALP, Alpaca; BC, Bactrian camel; CM, Conventional microscopy; DC, Dromedary camel; ELISA, Enzyme-linked immunosorbent assay; NA, not available; ND, not determined; PCR, Polymerase chain reaction; PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism.

[a]Selected positive samples. No prevalence data are available.

[b]Species-assignment based on morphological differences on the detected *Cryptosporidium* oocysts.

[c]Synonym of CC1.

[d]Synonym of EbpC.

those microeukaryotic parasites might act as potential sources of human cryptosporidiosis, giardiasis, and microsporidiosis (25, 47). To bridge this knowledge gap, this study aims to assess the presence, genetic diversity, and zoonotic potential of *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi* in dromedary camels with and without diarrhea in Aswan, the southernmost governorate in Upper Egypt.

2. Materials and methods

2.1. Study area and sampling

A total of 102 individual fecal samples from dromedary camels were collected in three geographical areas (Abu Simbel, Edfu, and Kom Ombo) of the Aswan Governorate, Upper Egypt (Figure 1). The calculation of the sample size was performed as described elsewhere (38) based on a 95% confidence level. Fecal samples were collected during the period from August to December 2021. Local farmers were approached and encouraged to participate in the study after their agreement with the study's goals and procedures. Once permission was granted, fecal samples were directly collected from the rectum of the animals and placed into a sterile polystyrene plastic flask containing 70% ethanol as a preservation agent. Basic epidemiological information (geographical origin, sex, age, fecal consistency, and physical condition) was collected at the time of sampling. Animals were reared in an open system under conventional pasture grazing dependent on grazing food including hay and forages. In winter, camels were partly fed on natural grazing, but feeding was complemented by food crops gathered by breeders, and grains may have been added to the diet in certain episodes of production. Out of the 102 samples collected, 26 were diarrheic and 76 formed. Samples were delivered to the Department of Zoonoses, Faculty of Veterinary Medicine (Sohag University, Egypt) and stored at 4°C. Samples were subsequently transferred to the Parasitology Reference and Research Laboratory of the National Center for Microbiology (Majadahonda, Spain) for downstream molecular studies.

2.2. DNA extraction and purification

Genomic DNA was isolated from ~200 mg of each fecal sample using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with the exception that samples combined with InhibitEX buffer were incubated for 10 min at 95°C. DNA samples were extracted and purified before being eluted in 200 µl of PCR-grade water and stored at 4°C until further molecular analysis. A maximum of 18 weeks elapsed between sample collection and DNA extraction.

2.3. Molecular detection and characterization of *Cryptosporidium* spp.

The presence of *Cryptosporidium* spp. was assessed using a nested-PCR protocol to amplify a 587-bp fragment of the *SSU* rRNA gene of the parasite (91). Approximately 3 µl of the DNA sample and 0.3 µM of the primer pairs CR-P1/CR-P2 in the primary reaction and CR-P3/CPB-DIAGR in the secondary reaction were used in the amplification procedures (50 µl) (Supplementary Table 1). Both PCR reactions were carried out as follows: one step of 94°C for 3 min, followed by 35 cycles of 94°C for 40 s, 50°C for 40 s, and 72°C for 1 min, concluding with a final extension of 72°C for 10 min.

Cryptosporidium parvum isolates were sub-typed by amplifying an 870-bp fragment of the *gp60* locus using a nested PCR (92). Reaction mixtures (50 µl) contained 2–3 µl of template DNA and 0.3 µM of the primer pairs AL-3531/AL-3535 and AL-3532/AL-3534 in the primary and secondary reactions, respectively (Supplementary Table 1). The PCR protocol for the main reaction consisted of an initial step of 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 59°C for 45 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. The secondary PCR settings were similar to the initial PCR except for the annealing temperature, which was 50°C.

2.4. Molecular detection of *Giardia duodenalis*

Detection of *G. duodenalis* DNA was achieved using a real-time PCR (qPCR) method targeting a 62-bp region of the gene codifying the *SSU* rRNA of the parasite (93). Amplification reactions (25 µl) consisted of 3 µl of template DNA, 0.5 µM of each primer Gd-80F and Gd-127R, 0.4 µM of probe (Supplementary Table 1), and 12.5 µl TaqMan® Gene Expression Master Mix (Applied Biosystems, CA, USA). The parasite DNA was detected using a Corbett Rotor Gene™ 6000 real-time PCR system (QIAGEN, Hilden, Germany) with an amplification protocol consisting of an initial hold phase of 2 min at 55°C and 15 min at 95°C followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Samples with qPCR cycle threshold values <32 were re-analyzed at the glutamate dehydrogenase (*gdh*) (94), β-giardin (*bg*) (95), and triose phosphate isomerase (*tpi*) (96) markers using specific PCR protocols to attempt to identify their assemblages and sub-assemblages.

2.5. Molecular detection and characterization of *Enterocytozoon bieneusi*

Detection of *E. bieneusi* was conducted by a nested PCR protocol to amplify the ITS region as well as portions of the flanking large and small subunits of the ribosomal RNA gene, as previously described (97). The outer EBITS3/EBTIS4 and inner EBITS1/EBITS2.4 primer sets (Supplementary Table 1) were used to generate PCR products of 435 and 390 bp, respectively. The main PCR was cycled at 94°C for 3 min, followed by 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and elongation at 72°C for 40 s), with a final extension at 72°C for 10 min. Conditions for the secondary PCR were identical to the primary PCR, except that only 30 cycles were performed at an annealing temperature of 55°C.

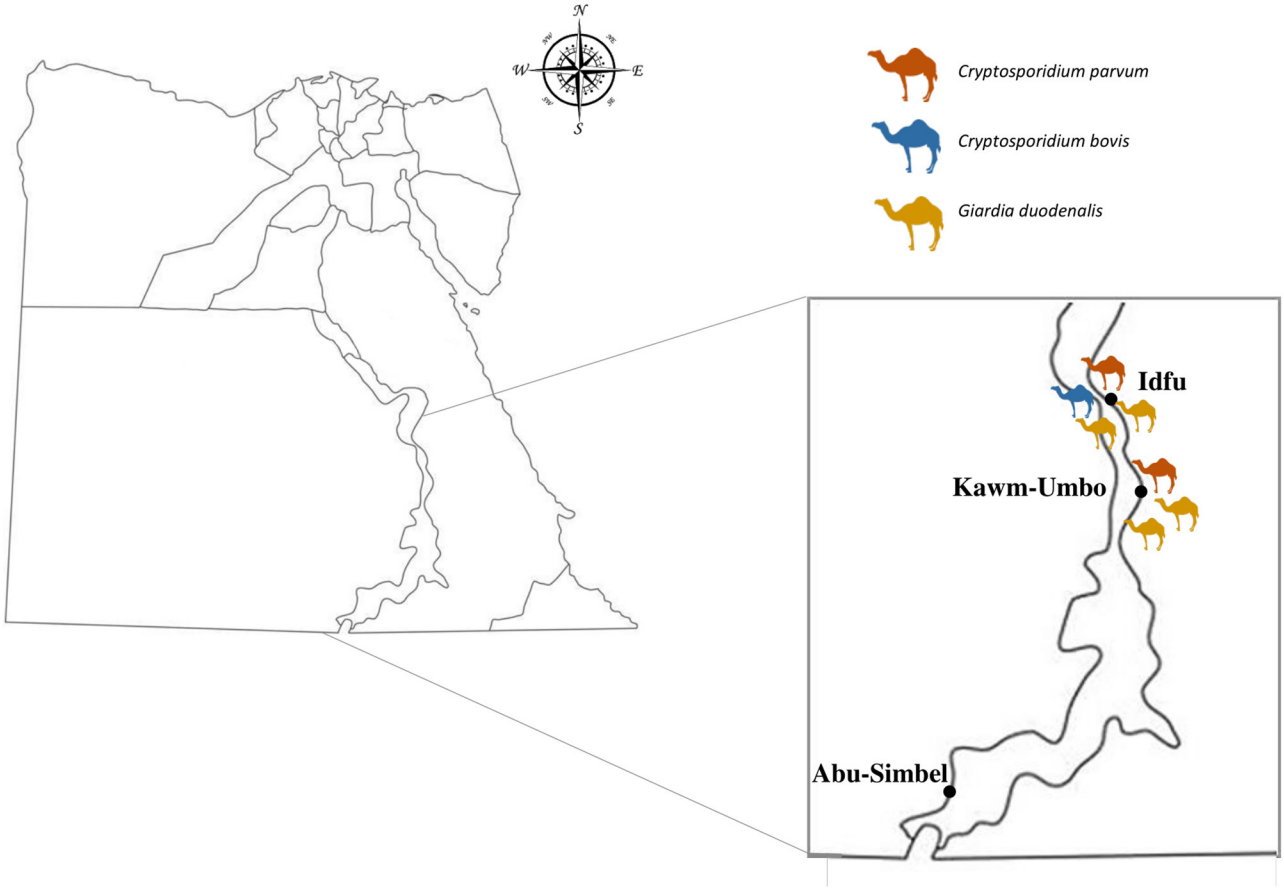

FIGURE 1
Map of Egypt showing the location of the sampling areas and the distribution of dromedary camels positive to *Cryptosporidium* spp. and *G. duodenalis*.

2.6. PCR and gel electrophoresis standard procedures

All of the aforementioned direct and nested PCR protocols were conducted on a 2720 Thermal Cycler (Applied Biosystems). Reaction mixes always included 2.5 units of MyTAQ^{TM} DNA polymerase (Bioline GmbH, Luckenwalde, Germany), and 5–10 µl of MyTAQ^{TM} Reaction Buffer with 5 mM dNTPs and 15 mM $MgCl_2$. For each parasite species studied, laboratory-confirmed positive and negative DNA samples of human and animal origin were routinely used as controls and included in each round of PCR. PCR amplicons were visualized on 1.5% D5 agarose gels (Conda, Madrid, Spain) stained with Pronasafe (Conda) nucleic acid staining solutions. A 100-bp DNA ladder (Boehringer Mannheim GmbH, Baden-Wurttemberg, Germany) was used to size the obtained amplicons.

2.7. Sequence analyses

Positive-PCR products of the expected size were directly sequenced in both directions using appropriate internal primer sets (Supplementary Table 1). DNA sequencing was conducted by capillary electrophoresis using the BigDye® Terminator chemistry (Applied Biosystems) on an ABI PRISM 3130 automated DNA sequencer. Generated DNA consensus sequences were aligned to appropriate reference sequences using MEGA6 (98) for species confirmation and genotype identification. The sequences obtained in this study have been deposited in GenBank under accession numbers OP365100 (*C. bovis*) and OP365101–OP365102 (*C. parvum*).

2.8. Statistical analyses

Fisher's exact tests were used to assess the relationships between parasitic infections and the different independent factors addressed in the study (geographical origin, sex, age, fecal consistency, and physical condition). A *P*-value of < 0.05 was considered statistically significant. Analyses were conducted using the statistical package SPSS version 25 (IBM Corporation, Armonk, NY, USA).

3. Results

3.1. Occurrence of the parasites

Giardia duodenalis was the most prevalent species found (3.9%, 4/102; 95% CI: 1.1–9.7), followed by *Cryptosporidium* spp. (2.9%,

3/102; 95% CI: 0.6–8.4). In contrast, *E. bieneusi* DNA was not detected in the dromedary camel population under investigation. The distribution of the *Cryptosporidium* and *G. duodenalis* infections according to the variables considered in the study is shown in Table 2. *Cryptosporidium* infections were detected in male animals younger than 5 years age from Edfu and Kom Ombo localities. Two of the three infections were detected in animals that had diarrhea. One of the three cryptosporidiosis-infected animals had emaciation, weakness, and roughened skin. *Giardia* infections were also detected in male dromedary camels only from Edfu and Kom Ombo localities. In contrast to *Cryptosporidium*, all *Giardia* infections were found in animals older than 5 years of age, primarily without diarrhea and in good physical condition. None of the three intestinal protist species proved positive in the dromedary camels sampled at Abu Simbel.

3.2. Risk association analyses

Dromedary camels younger than 2 years were significantly more likely to be infected by *Cryptosporidium* spp. than animals of older age ($P < 0.05$). None of the remaining variables were associated with an increased risk of infection by *Cryptosporidium* spp. or *G. duodenalis*.

3.3. Molecular data

The results of the *Cryptosporidium* sequencing analysis generated in the present study are summarized in Table 3. One of the three *Cryptosporidium*-positive samples was identified as cattle-adapted *C. bovis*, showing 100% identity with a stretch of 455 bp from position 315–770 of reference sequence AY741305. The remaining two samples were recognized as zoonotic *C. parvum*, and their sequences varied from reference sequence AF112571 by four to five single nucleotide polymorphisms (SNPs), including a TAAT deletion at positions 686–689 of AF112571. During the visual assessment of chromatograms, no ambiguous positions in the form of double peaks were found. Attempt to amplify the *C. parvum* isolates at the *gp60* locus failed, so the subtype family of the parasite remained unknown.

All four *G. duodenalis*-positive isolates yielded C_T values >32 (median: 35.9; range: 32.8–38.5) at qPCR, indicating a relatively low quantity of parasite DNA in the original samples. None of these samples could be amplified at the *gdh*, *bg*, and *tpi* loci.

4. Discussion

This study adds to the body of knowledge about the occurrence and genetic diversity of the diarrhea-causing intestinal protists *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi* in Egyptian dromedary camels. The main strength of the survey is the use of PCR and Sanger sequencing technologies, allowing for accurate detection, differentiation, and characterization of the investigated pathogens. The survey is also relevant because (i) it focuses on a host species (dromedary camel) for which parasite epidemiological data are particularly scarce in Egypt, (ii) it demonstrates that dromedary camels can act as the potential source of human cryptosporidiosis caused by *C. parvum*, and (iii) information gathered is useful for developing proper intervention and control strategies against oral–fecal transmitted diseases, including cryptosporidiosis and giardiasis (79, 99).

Cryptosporidium infections were detected in 3% of the investigated dromedary camels. Surprisingly, its incidence percentage was lower (4–24%) than those detected by conventional microscopy in other Egyptian camel populations (33–37, 39, 40). However, a slightly superior rate of 6% was reported in a similar study conducted using PCR-RFLP (38). These disparities between microscopy and PCR data might be attributed to fundamental epidemiological (infection pressure and geographical area) and host (age and immunological state) differences among the camel populations surveyed. However, unwanted false-positive results are prevalent during microscope investigation and might lead to overestimated prevalence rates (100). Similar highly variable *Cryptosporidium* prevalences have been observed by conventional microscopy or ELISA techniques in various Middle Eastern countries, including Iran (2–100%), Iraq (7–100%), and Saudi Arabia (15–22%; see Table 1). Our genotyping data revealed the presence of two *Cryptosporidium* species, including *C. parvum* (in two animals presenting with diarrhea) and *C. bovis* (in an asymptomatic animal). *Cryptosporidium* infections have been previously reported in diarrheic dromedary camels in Algeria (27) and Iran (48), whereas *C. parvum* has already been described in Egyptian dromedary camels (38); this is the first report of cattle-adapted *C. bovis* in this host species in the country and the third report globally after the description of the parasite in Bactrian camels in China (61, 62). In Egypt, previous research has revealed the occurrence of *C. bovis* in cattle and buffalo populations (101–105). These findings show that *C. bovis* cross-species transmission is likely in areas where different domestic ruminant species share habitat. Although the two dromedary camels infected with this *Cryptosporidium* species manifested diarrhea, we were unable to amplify the two *C. parvum* isolates at the *gp60* locus. The lack of diagnostic data for viral or bacterial agents was an obstacle to unambiguously linking the occurrence of diarrhea with a given enteric pathogen. In this regard, light *C. parvum* infections associated with modest oocyst shedding might explain the amplification failure at the single copy *gp60* gene, a marker known for its limited diagnostic sensitivity (21). Notably, *C. parvum gp60* genotype families IIa and IId have been found in Egyptian dromedary camels (38). It should be stressed that *C. parvum* is regarded as a common zoonotic *Cryptosporidium* species with loose host specificity and worldwide distribution, whereas human cases of cryptosporidiosis caused by *C. bovis* are sporadically reported globally (22, 23). Therefore, our molecular data support the potential zoonotic spread of those *Cryptosporidium* species between infected dromedary camels and humans.

In the present study, *G. duodenalis* was the predominant (4%) protozoan parasite found among the examined camel population. Conventional microscopy revealed a fairly comparable *G. duodenalis* infection rate of 5% in the sole prior investigation undertaken on this host species in Egypt (37). Epidemiological information on camel populations in other Middle Eastern

TABLE 2 Distribution of *Cryptosporidium* spp. and *Giardia duodenalis* infections according to geographical origin, sex, age, fecal consistency, and physical condition of examined camels ($n = 102$).

Variable	Total (*n*)	*Cryptosporidium* spp.			*Giardia duodenalis*		
		Infected (*n*)	%	*P*-value	Infected (*n*)	%	*P*-value
Geographical origin							
Abu-Simbel	35	0	0	0.53	0	0	0.46
Idfu	35	2	5.7		2	5.7	
Kawm-Umbo	32	1	3.1		2	6.2	
Sex							
Male	95	3	3.2	1	4	4.2	1
Female	7	0	0		0	0	
Age (yrs.)							
≤2	13	2	15.4	0.01*	0	0	1
2–5	15	1	6.7		0	0	
≥5	74	0	0		4	5.4	
Diarrhea							
Yes	26	2	7.7	0.17	1	3.8	1
No	76	1	1.3		3	4	
Physical condition							
Normal	84	2	2.4	0.45	4	4.8	1
Emaciated	18	1	5.6		0	0	

Statistically significant values are highlighted in bold with a star (Fisher exact test was used).
NS, not statistically significant at the Fisher exact test.
*P-value < 0.05: statistically significant.

TABLE 3 Frequency and molecular diversity of *Cryptosporidium* spp. identified in camels in the present study.

Species	No. isolates	Locus	Reference sequence	Stretch	Single nucleotide polymorphisms	GenBank ID
C. bovis	1	*SSU* rRNA	AY741305	315–770	None	OP365100
C. parvum	1	*SSU* rRNA	AF112571	544–983	A646G, T649G, 686_689delTAAT, and T693A	OP365101
C. parvum	1	*SSU* rRNA	AF112571	527–1,030	A646G, T649G, 686_689delTAAT, T693A, and T972A	OP365102

del, Deletion; *SSU* rRNA, Small subunit ribosomal RNA.

countries is also scarce and completely absent in African countries other than Egypt. Prevalence rates of 4–24% have been documented in Iraq (51, 53, 54). The parasite is also known to be circulating at an unknown infection rate in dromedary camels in Saudi Arabia (73). All the previously mentioned studies were based on conventional microscopy, so information on the *G. duodenalis* assemblages and sub-assemblages causing the infections is also lacking. It is noteworthy that *G. duodenalis* has been detected at occurrence rates of 7–10% in Chinese Bactrian camels by PCR (61, 64, 65, 74). All these infections were caused primarily by ungulate-adapted *G. duodenalis* assemblage E and, to a lesser extent, by zoonotic *G. duodenalis* assemblage A (see Table 1). In our study, the four *G. duodenalis*-positive samples (three in asymptomatic animals and one in a diarrheic animal) yielded high C_T values (>32) at qPCR and impeded the completion of genotyping analyses at appropriate genetic markers, including the genes encoding for the glutamate dehydrogenase (*gdh*), beta-giardin (*bg*), and triosephosphate isomerase (*tpi*) proteins of the parasite. As in the case of the *Cryptosporidium gp60* locus, the *Giardia gdh*, *bg*, and *tpi* loci are single-copy genes with limited diagnostic sensitivities, making them unsuitable for amplifying samples with a small amount of parasitic DNA. The high C_T values obtained at qPCR are also indicative of light infections, compatible with the absence of gastrointestinal manifestations (diarrhea) in most *Giardia*-positive dromedary camels. The lack of genotyping data at the assemblage and sub-assemblage levels does not allow us to fully assess the zoonotic implications of our findings. More research should be conducted to ascertain the genetic diversity of *G. duodenalis* infections in camels and their role as potential sources of human giardiasis.

No DNA of the microsporidia *E. bieneusi* could be detected in any of the fecal DNA samples analyzed in the present study, suggesting that dromedary camels are not relevant hosts in the transmission of this pathogen in Egypt. Very few epidemiological

studies have attempted to investigate the occurrence and genetic diversity of *E. bieneusi* in camels globally. In the only survey conducted in Africa to date, a PCR prevalence rate of 20% was estimated in Algerian dromedary camels (25). In that study, two *E. bieneusi* genotypes were detected, including Camel-2 and Macaque1. More information is available from Bactrian camels in China, where *E. bieneusi* seems to be a common finding with infection rates in the range of 30–45% (61, 64, 65, 75). Most of the infections detected were caused by camel-adapted *E. bieneusi* genotypes, including CAM1 to CAM6, but the presence of genotypes such as BEB6, EbpA, EbpC, and O (all four members of phylogenetic Groups 1 and 2, including zoonotic genetic variants of the parasite) indicate that Bactrian camels can serve as potential sources of *E. bieneusi* infections to humans (77, 78). It should be noted that, in Egypt, *E. bieneusi* has been previously detected in immunosuppressed patients with and without diarrhea (106, 107), in children attending day-care centers (108), and in domestic animals including cattle, buffaloes, rabbits, sheep, goats, cats, and dogs (109). These data highlight the need to investigate the role of other animal host species (including dromedary camels) as potential sources of human microsporidiosis by *E. bieneusi* in the country.

Regarding the analysis of variables potentially associated with an increased risk of infection by enteric protists, dromedary camels younger than 2 years of age were more likely to be infected by *Cryptosporidium* spp., this being the only statistically significant association found in the present study. This result is consistent with those obtained in a study that found greater *Cryptosporidium* infection rates in 1-year-old camel calves than in older animals in Iran (48). Discrepant results have been reported in other surveys. For instance, *Cryptosporidium* infections were more frequently identified in camels in the age groups of 1–4 years in Algeria (27) and 3–6 years in Iraq (51). A third study that was conducted in Iran revealed no significant associations between camel age and *Cryptosporidium* infection status (44). Although not statistically significant, all dromedary camels sampled at Abu Simbel tested negative for *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi*, suggesting that environmental (e.g., geographical area of origin and local climatic conditions) and biological (e.g., host age and immunological status) conditions and management practices (e.g., contact with other livestock) might play a role in the occurrence and distribution of these pathogens. Taken into account, most of the studied animals were reared in resource-poor settings, including water and food sources, which, together with the management practices, affect the occurrence of the reported pathogens. A lack of access to safe drinking water and poor sanitation and hygiene practices were identified as potential factors linked with a higher risk of developing diarrhoeal illness (15). In relation to feeding habitat, several previous studies have revealed an obvious association between the occurrence of various parasites in camels and grazing performance, including bushes and grasses. In this respect, logging of shrubs, bushes, and trees for rain-fed production systems might enhance the probability of harvesting the ova and/or larvae from pasture (110). Given the above findings, our study pointed out that the application of strict control and hygienic measures represented by providing clean drinking water, improvement of sanitation and hygiene practices are mandatory preventive strategies to control these zoonotic pathogens. Furthermore, regular administration of antiparasitic drugs and treatment of infected camels in the studied area stand as major control measures for the infection and should be adopted, together with the strict quarantine of imported animals from neighboring regions.

Some design and methodological limitations might have biased the accuracy of the results obtained in the present study and should be considered when interpreting them. First, the smaller sample size may have led to underestimating true prevalence rates and lowered the power of the statistical analyses conducted. Second, the transversal nature of the study might not be adequate to capture potential temporal/seasonal variations in parasite occurrence. Third, the animal population under study was mainly composed of adult animals, which are less likely to be infected by the diseases studied. Fourth, suboptimal fecal sample storage and transportation conditions might have altered the quantity and quality of the DNA used for diagnostic and genotyping purposes. Fifth, the lack of genotyping data for some of the protist species investigated (e.g., *G. duodenalis*) made it difficult to fully analyze the epidemiological and zoonotic implications of our findings.

5. Conclusion

This is one of the very few molecular-based epidemiological studies aiming at investigating the presence and molecular diversity of diarrhea-causing enteric protist parasites in dromedary camels in African countries, including Egypt. *Cryptosporidium* spp. and *G. duodenalis* were identified at low (<5%) infection rates. Sequence analyses revealed the presence of two *Cryptosporidium* species, including zoonotic *C. parvum* and cattle-adapted *C. bovis*. This is the first report of *C. bovis* in dromedary camels globally. The presence of *C. parvum* implies that dromedary camels play a role in the transmission of this *Cryptosporidium* species and can serve as potential sources of human cryptosporidiosis. Implementation of stricter hygienic measures and awareness raising are recommended to minimize the zoonotic hazard of camel pathogens to people in contact with these animals or their manure. Improving water and food resources in the studied area seems mandatory to reduce the transmission of infection by these zoonotic pathogens. Further research is warranted to corroborate and expand these preliminary findings in larger camel populations and other animal species in Upper Egypt.

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 authors.

Ethics statement

The animal study was reviewed and approved by the Research Ethics Committee of the Faculty of Veterinary Medicine, Sohag University (Egypt) on 01.12.2019. Written informed consent was

obtained from the owners for the participation of their animals in this study.

Author contributions

EE, SA, AS, AG, ML, BA, and EH collected the samples. EE, PK, AD, CH-C, and BB conducted laboratory experiments. PK and AD conducted sequence analyses. EE conducted statistical analyses. SA and ML secured the funding for conducting sampling and experimental work. EE, DG-B, and DC designed and supervised the experiments. EE and DC writing—original draft preparation. EE, SA, AG, AS, AD, DG-B, and DC writing—review and editing. The final version was read and approved by all authors.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2023.1139388/full#supplementary-material

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Isolation and genetic characterization of MERS-CoV from dromedary camels in the United Arab Emirates

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Background: The study of coronaviruses has grown significantly in recent years. Middle East respiratory syndrome coronavirus (MERS-CoV) replicates in various cell types, and quick development has been made of assays for its growth and quantification. However, only a few viral isolates are now available for investigation with full characterization. The current study aimed to isolate MERS-CoV from nasal swabs of dromedary camels and molecularly analyze the virus in order to detect strain-specific mutations and ascertain lineage classification.

Methods: We isolated the virus in Vero cells and adapted it for *in vitro* cultivation. The isolates were subjected to complete genome sequencing using next-generation sequencing followed by phylogenetic, mutation, and recombination analysis of the sequences.

Results: A total of five viral isolates were obtained in Vero cells and adapted to *in vitro* cultures. Phylogenetic analysis classified all the isolates within clade B3. Four isolates clustered close to the MERS-CoV isolate camel/KFU-HKU-I/2017 (GenBank ID: MN758606.1) with nucleotide identity 99.90–99.91%. The later isolate clustered close to the MERS-CoV isolate Al-Hasa-SA2407/2016 (GenBank ID: MN654975.1) with a sequence identity of 99.86%. Furthermore, the isolates contained several amino acids substitutions in ORF1a (32), ORF1ab (25), S (2), ORF3 (4), ORF4b (4), M (3), ORF8b (1), and the N protein (1). The analysis further identified a recombination event in one of the reported sequences (OQ423284/MERS-CoV/dromedary/UAE-Al Ain/13/2016).

Conclusion: Data presented in this study indicated the need for continuous identification and characterization of MERS-CoV to monitor virus circulation in the region, which is necessary to develop effective control measures. The mutations described in this investigation might not accurately represent the virus's natural evolution as artificial mutations may develop during cell culture passage. The isolated MERS-CoV strains would be helpful in new live attenuated vaccine development and efficacy studies.

KEYWORDS

MERS-CoV, coronavirus, isolation, characterization, dromedary, camel, zoonotic disease

1. Introduction

Middle East respiratory syndrome (MERS) is a viral respiratory infection of humans and dromedary camels that is caused by a coronavirus called the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) (1). During the last two decades, the world witnessed the emergence of three novel coronaviruses: severe acute respiratory syndrome coronavirus 1 (SARS CoV-1) in 2002–2003, Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012, and the ongoing pandemic caused by SARS-CoV-2 in 2019, resulting in a very significant impact on both humans and animals as well as the global economy. Coronaviruses can have a devastating impact on the health of humans and animals. Coronaviruses are important RNA viruses, and members of the subfamily Orthocoronavirinae are classified into four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus (2, 3).

From April 2012 to July 2022, a total of 2,591 laboratory-confirmed cases of Middle East respiratory syndrome (MERS) were reported globally, with 894 associated deaths at a case-fatality ratio (CFR) of 34.5% (4). Via direct or indirect contact, humans can become infected with the MERS-CoV from dromedary camels (*Camelus dromedarius*), the virus's natural host and zoonotic source. MERS-CoV has been identified in dromedaries in several countries in the Middle East, Africa, and South Asia. Some dromedary camels have shown minor upper respiratory symptoms related to MERS-CoV. While MERS-CoV has little effect on animal health, human infections adversely affect public health. MERS is an emerging disease with severe implications for human public health. MERS-CoV phylogeny currently comprises three major clades, provisionally named clades A, B, and C. The clade B strains currently infect humans and dromedary camels in the Arabian Peninsula, in contrast to clades A and C, which contain extinct strains and strains that are not circulating in this region. Clade B is divided into six phylogenetic lineages. In dromedary camels, the recombination between lineages 3 and 4 led to the emergence of the circulating recombinant lineage 5 around the year 2014 (5–8). More than 41,000 nasal swabs taken from camels in the United Arab Emirates (UAE) between 2013 and 2021 were tested for MERS-CoV by quantitative reverse transcription polymerase chain reaction (RT-qPCR) at the veterinary laboratories of Abu Dhabi Agriculture and Food Safety Authority (ADAFSA). Additionally, we conducted numerous research on various aspects of MERS-CoV infection, including the evaluation of diagnostic tests, epidemiology, and investigation of infection events in humans linked to sick camels, identification of the diversity of the virus in dromedary camels, zoonotic origin and transmission of the virus, and risk factors for the virus seropositivity among animal market and slaughterhouse workers (8–12).

This study aimed to isolate MERS-CoV from nasal swabs of dromedary camels, and propagate and molecularly characterize the isolated virus. The newly isolated virus serves as a good starting point for developing diagnostic tests and inactivated vaccines.

2. Materials and methods

2.1. Study animals

Dromedary camels were sampled at an open-air animal market in Al Ain, United Arab Emirates for MERS-CoV between September 2016 and November 2017 to investigate risk factors and genetic diversity of the virus. A nasal swab was taken from each of the 372 sampled camels together with information on age, sex, and animal origin. In order to minimize risk when handling MERS-CoV-suspected camel specimens and ensure correct specimen management, prompt communication between ADAFSA collecting veterinarians and laboratory staff was set up.

2.2. Sample collection and processing

Nasal samples were obtained by using a Dacron swab kit containing Puritan UniTranz-RT 1 mL Universal Transport Solution (Puritan, Brescia, Italy). All samples were barcoded and then transported to the third-level biological safety laboratory (BSL3) of ADAFSA in Abu Dhabi within 8 h using cool boxes maintained between 4–8°C. Each sample was mixed by pulse-vortexing for 15 s, the solution was transferred to a sterile 5 mL cryovial, and the swab kit was autoclaved and discarded. Samples were centrifuged at 1500 rpm for 10 min at 4°C, and the supernatants were filtered with a 0.45 µ filter (Millipore, Billerica, MA, United States).

2.3. Nucleic acid extraction and RT-qPCR

A volume of 400 µL from each nasal swab was used for the nucleic acid extraction with the EZ1 Virus Mini Kit v2.0 (48) kit (Qiagen), and the final elution was done in 60 µL. Screening for MERS-CoV was performed using the Coronavirus MERS-CoV RT-PCR (ModularDx Kit Coronavirus SA1 (EMC) upstream E-gene and Light Mix Modular MERS-CoV Orf1a), targeting the upstream region of the envelope gene (upE) and open reading frame 1a (ORF1a) (13, 14). The RT-qPCR was performed on LightCycler 2.0 using Roche's LightCycler RNA Virus Master Chemistry (Basel, Switzerland).

2.4. Virus isolation

All the nasal swab samples were tested for MERS-CoV by RT-qPCR. Only samples with Cp (cycle threshold) values below 22 were subsequently used for virus isolation. A total of 10 nasal swabs positive by PCR were inoculated into Vero E6 cells (African green monkey kidney) obtained initially from American Type Culture Collection (ATCC, Rockville, MD, United States) specimen CCL-81™. Cells were maintained in Dulbecco's modified Eagle's medium with 4.5 g/L D-glucose, and l-glutamine (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, United States) and 5 mL of Gibco Antibiotic-Antimycotic solution (containing 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Amphotericin B).

We inoculated Vero E6 cells seeded at 80–90% confluence in 6-well cell culture plates (Corning Inc., United States) with 100 µL of the samples and incubated the cells at 37°C in a 5% carbon dioxide atmosphere. A control well inoculated with sterile phosphate-buffered saline (PBS) was included on each plate. Plates were centrifuged at 1000 rpm (18 × g for 60 min) using a small benchtop centrifuge in a sealed biocontainment bucket (SL 8R Small Benchtop Centrifuge, Thermo Scientific, Germany). This system works because the

low-speed centrifugation enhances viral adsorption to the susceptible cells. It is thought that the minor trauma to the cell surface produced because of low-speed centrifugation mechanical force enhances the viral entry into the cells, reducing the total time taken for the virus to produce infection of cells (15).

The inoculum was removed, cells were gently washed three times with DMEM, and a fresh medium was added. The infected cells were monitored daily for 7 days to check for cytopathic effects (CPE) using an inverted microscope, and the medium was changed on alternate days. When 70–90% CPE was observed, the supernatant and cells were harvested by freezing and thawing three times, and confirmation of virus isolation was done by RT-qPCR as described above. For those inoculated samples that showed no CPE, a blind passage was made using the freeze–thaw method. If no CPEs were observed until three successive blind passages, the sample was considered negative. Subsequent passages were performed in T-25 flasks.

2.5. Whole genome sequencing

2.5.1. Nucleic acid extraction and sequence-independent, single-primer-amplification

The cell culture extracts (400 µL) of MERS-CoV positive samples were used for the nucleic acid extraction with the EZ1 Virus Mini Kit v2.0 (48) kit (Qiagen). The final elution was done in 60 µL.

Random amplified cDNA was prepared for each sample using an approach that was previously described in another study (16). For reverse transcription, 4 µL of RNA and 1 µL of primer A (5′-GTTTCCCACTGGAGGATA-N9-3′, 40 pmol/µL) were mixed and incubated for 5 min at 65°C followed by cooling to room temperature. To synthesize the first-strand, the volumes of 2 µL SuperScript IV first-strand buffer, 1 µL of 12.5 mM dinucleoside triphosphates (dNTPs), 0.5 µL of 0.1 M dithiothreitol (DTT), 1 µL H_2O, and 0.5 µL SuperScript IV (Thermo Fisher) were mixed and incubated for 10 min at 42°C. The following substances were mixed to synthesize the second strand: 1 µL Sequenase buffer, 0.85 µL H_2O, and 0.15 µL Sequenase (Affymetrix) and incubated at 37°C for 8 min, followed by the addition of 0.45 µL Sequenase dilution buffer and 0.15 µL Sequenase. The mixture was incubated for 8 min at 37°C. Amplification of cDNA was performed in triplicate using 5 µL of the reaction mixture as input to a 50 µL of LA Taq (Sigma) reaction mixture, according to the manufacturer's instructions, using 1 µL primer B (5′-GTTTCCCACTGGAGGATA-3′). The amplification conditions consisted of 98°C for 30 s, and 30 cycles of 94°C for 15 s, 50°C for 20 s, and 68°C for 2 min followed by 68°C for 10 min. The amplified cDNA was purified using AMPure XP beads (Beckman Coulter, Brea, CA) and quantified with a Qubit high-sensitivity double-stranded DNA (dsDNA) kit (Thermo Fisher) following the manufacturer's instructions.

2.5.2. MiSeq™ library preparation and sequencing

Amplified Round B cDNA from all samples were purified using AMPure XP beads (Beckman Coulter), and 2 ng was used as input into the Nextera XT kit (Illumina) previously described (17). After 13 cycles of amplification, Illumina library concentration and average fragment size were determined using the Agilent 4,200 TapeStation System. Sequencing was performed on an Illumina MiSeq using a V3 MiSeq™ (600 cycles) Reagent kit (Illumina, San Diego, CA, United States).

2.5.3. Long read sequencing

The cDNA of the sample with GenBank ID: OQ423284 (Lab ID: VL_13) and the sample with GenBank ID: OQ423287 (Lab ID: VL_765) were further subjected to long-read sequencing. The library was prepared using Ligation Sequencing Kit (SQK-LSK109), and samples were barcoded with Native Barcoding Expansion 1–12 (EXP-NBD104). Finally, 30 ng of the prepared library was sequenced on a Nanopore MinION device using the R9.4.1 flow cell.

2.5.4. Bioinformatics analysis

The Illumina sequences were first evaluated for their quality using FastQC software,[1] and the low-quality reads were trimmed with trimmomatic software[2] using the following settings: TRAILING:28 SLIDINGWINDOW:4:15 MINLEN:35 to remove the reads with quality below 15 or length less than 35 bp. The adaptors were removed from long reads using the Porechop tool.[3]

De novo assembly of short reads was performed with the shovill pipeline[4] with a command: shovill --outdir out --R1 path/to/read1.fasta --R2 path/to/read2.fastq. The accuracy of the assembly was evaluated with the Quast tool[5] with a command: quast -o assembly/quast path/to/scaffolds.fasta. The hybrid assembly for samples (GenBank IDs: OQ423284 and OQ423287) was accomplished with Spades.[6] The consensus sequence of the final assembly was first analyzed by BLAST services, followed by phylogenetic analysis. The variant calling against the MERS-CoV reference Saudi isolate JX869059 (EMC/2012) was performed with the snippy tool with an option --mincov 20 (minimum coverage of 20). The nucleotide genome sequences obtained in this study were deposited in GenBank under the accession numbers OQ423283 to OQ423287.

2.5.5. Phylogenetic analysis of MERS-CoV

The BLAST tool available in the NCBI was used for each strain to determine the most closely related sequences of MERS-CoV, and the corresponding sequences, along with the newly isolated sequences, were then used to construct a phylogenetic tree. The final alignment contained 118 sequences from a nearly complete genome (~30,000 nt in length), including human cases of animal origin. The multiple sequence alignment was performed with the ClustalW tool, and the tree was constructed with the Maximum Likelihood method (ML) and Kimura 2-parameter model (18) with 1,000 Bootstrap confidence using MEGA X software (19).

2.5.6. Recombination analysis using RDP5

The Recombination Detection Program (RDP5) v.5.3 (20) was used to detect the possible recombination events using the default settings of algorithms GENECONV, BOOTSCAN, MaxChi, Chimaera, and 3Seq implemented in the RDP5 program. The

1 https://github.com/s-andrews/FastQC
2 https://github.com/usadellab/Trimmomatic
3 https://github.com/rrwick/Porechop
4 https://github.com/tseemann/shovill
5 https://github.com/ablab/quast
6 https://github.com/ablab/spades

sequences from this study were aligned with the reference sequences of MERS-CoV representing different clades (~30.000 bp were used for phylogenetic analyses), and the recombination events, likely parental isolates of recombinants, and recombination breakpoints were analyzed.

3. Results

3.1. Virus isolation

After 3 days, we observed virus-induced cytopathic effects (CPE) in three samples, and on the first blind passage, two more isolates were obtained. Table 1 provides information on the five virus isolates and the dromedary camels used for sample collection. Cells infected with all five isolates showed focal rounding and swelling of cells from the fourth day of inoculation, followed by detachment, plaque, and syncytium formation (Figure 1). The presence of MERS-CoV was confirmed by RT-qPCR.

3.2. Phylogenetic analysis

A phylogenetic analysis using nearly entire genome sequences (30.000 bp) of 118 MERS-CoV sequences, including our isolates, was conducted to determine which group and lineage our isolates belonged to. The result showed that all five isolates clustered within clade B3 with variable relatedness to the previous UAE isolates. The samples with GenBank IDs OQ423283, OQ423285, OQ423286, and OQ423287 formed a separate cluster and were close to the MERS-CoV isolate camel/KFU-HKU-I/2017 (GenBank ID: MN758606.1), as shown in (Figure 2). The sample GenBank ID: OQ423284 was clustered close to the MERS-CoV isolate Al-Hasa-SA2407/2016 (GenBank ID: MN654975.1), as shown in Figure 2.

The MERS-CoV isolates shared 99.69–99.99% nucleotide sequence intragroup identity, 99.73–99.91% identity with the MERS-CoV isolate camel/KFU-HKU-I/2017 (sequence ID: MN758606.1), 99.74–99.86% with the MERS CoV isolate Al-Hasa-SA2407/2016 (GenBank ID: MN654975.1), and 99.42–99.47% identity with the Human betacoronavirus 2c EMC/2012 (GenBank ID: JX869059.2) (Table 2).

3.3. Mutational analysis

The single nucleotide polymorphism (SNP) was called based on the original Saudi MERS-CoV reference isolate JXJX869059, where several mutations were reported. Some are unique for these isolates.

3.3.1. Missense variants

In coding region ORF1a, there were 32 missense variants, of which 18/32 were shared between the five isolates, 9/32 were found only in OQ423284, 1/32 was found in samples OQ423284 and OQ423285, 1/32 was found in samples OQ423283, OQ423285 and OQ423287, 2/32 were found in samples OQ423283, OQ423285, OQ423286 and OQ423287, and 1/32 was found in samples OQ423283, OQ423284, OQ423285, and OQ423287. In ORF1ab, there were 25 missense variants, of which 18/25 were shared between the five isolates, 4/25 were found in samples OQ423283, OQ423285, OQ423286 and OQ423287, 1/25 was found in samples OQ423283, OQ423284, OQ423285, and OQ423287, and 1/25 was found in samples OQ423283, OQ423284, OQ423285, and OQ423286. In the spike, there were two missense variants: one variant (p.Phe473Ser) was found in OQ423283 only, while the other (p.Gln1020Arg) was found in samples OQ423283, OQ423284, and OQ423285. In ORF3, there were four missense variants, and three variants (p.Val62Phe, p.Leu77Arg, and p.Pro86Leu) were shared with five samples. Another variant (p.Leu17Phe) was found in samples OQ423283, OQ423284, OQ423286, and OQ423287. In ORF4b, there were four missense variants, of which three variants (p.Met6Thr, p.Pro106Ser, and p.His44Pro) were shared with five samples. Another variant (p.Ala139Val) was found in OQ423283 only. In M, there were three missense variants, of which two variants

TABLE 1 Details on the five virus isolates and the dromedary camels from which samples were collected.

SN	Animal ID	Virology lab ID	Virus isolates	Date of sample collection	GenBank accession	Animal origin	Camel age (months)	Sex
1	1,060,295	VL-12	MERS-CoV/dromedary/UAE/Al Ain/12/2016	Oct. 16, 2016	OQ423283	Abu Dhabi	32	Female
2	1,060,297	VL-13	MERS-CoV/dromedary/UAE/Al Ain /13/2016	Dec. 12, 2016	OQ423284	Al Ain	12	Male
3	10,041,704	VL-704	MERS-CoV/dromedary/UAE/Al Ain /704/2017	Feb. 2, 2017	OQ423285	Abu Dhabi	12	Male
4	10,041,763	VL-763	MERS-CoV/dromedary/UAE/Al Ain /763/2017	Sept. 22, 2017	OQ423286	Abu Dhabi	8	Male
5	10,041,765	VL-765	MERS-CoV/dromedary/UAE/ Al Ain/765/2017	Nov. 11, 2017	OQ423287	Al Ain	10	Male

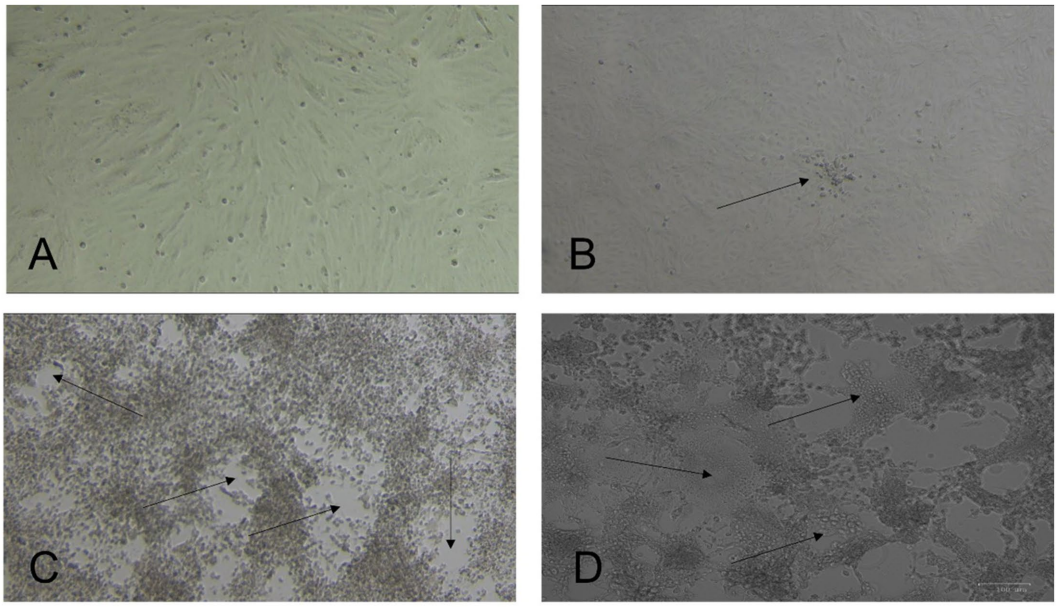

FIGURE 1
Isolation of Middle East respiratory syndrome coronavirus (MERS-CoV) from nasal swabs of dromedary camels. Vero E6 cells were incubated at 37°C and observed daily for cytopathic effect (CPE) under a light microscope. **(A)** Normal uninfected cells. **(B)** Early CPE of focal cell rounding at day three post-inoculation (PI) (arrow). **(C)** Extensive CPE and plaque formation (arrows) observed on day 5 PI. **(D)** Multinucleated giant cell formation (syncytial formation) (arrows). Original magnification ×10.

(p.Val69Ile and p.Phe123Ile) were found in all samples. Another variant (p.Ala84Ser) was found in OQ423284 only. In ORF8b, there was a single missense variant (p.Leu4Pro) detected in all samples. In N, there was a single missense variant (p.Gly198Ser) found in OQ423286 only. The summary of the missense mutations is shown in Table 3 and more details are provided in Supplementary Table S1.

3.3.2. Synonymous variants

In ORF1a, there were 46 synonymous variants, of which 25 were shared between the five isolates. In ORF1ab, there was only one synonymous variant shared between the isolates. There were 17 synonymous variants in S, of which 11 were found in all isolates. In ORF3, there were two synonymous variants. In ORF4a, there were two synonymous variants with one shared between the isolates. In ORF4b, there were four synonymous variants, with only one shared between the isolates. In ORF5, there were five synonymous variants, of which four were shared between the isolates. In M, one synonymous variant was shared between the isolates. In N, there were four synonymous variants. The summary of synonymous variants across the viral genome is provided in Supplementary Table S2.

3.4. Analysis of recombination

The recombination analysis indicated that OQ423284/MERS-CoV/dromedary/ UAE-Al Ain/13/2016 is a recombinant strain (Figure 3) with close similarity with B3.MN654975.1/*Camelus dromedarius*/KSA/isolate Al-Hasa-SA2407/2016 as a major parent (99.9%) and B3.MN 655008.1/*Camelus dromedarius*/KSA/isolate Al-Hasa-SA2696/2017 as a minor parent (99.9%), both of which are nearly identical. The recombination event was detected with six algorithms (RDP, GENECONV, BootScan, MaxiChi, Chimaera, and 3Seq). The beginning and end of the recombination event detected by each algorithm, along with the significant *p*-values, are shown in Table 4.

4. Discussion

MERS-CoV replicates in a wide range of cell types (23, 24) and *in vitro,* and assays for MERS-CoV growth and quantification have been rapidly developed. However, few virus isolates are currently available with full characterization for research work (25–29). Besides, the high mortality rate of ~34% and the continuous introduction of the virus from dromedary camels to humans urge for the establishment of research infrastructure to aid in research and development. Hence, in the current study, attempts were made to isolate the MERS-CoV from nasal swabs of dromedary camels. In the present study, five viral isolates were obtained from nasal swabs of dromedary camels in the UAE and then adapted to *in vitro* cultures and further molecularly characterized to identify strain-specific variations and to determine lineage classification.

The phylogenetic analysis performed in this study indicated that all five isolates belonged to clade B3 of MERS-CoV and were clustered close to the MERS-CoV isolated from camels in Saudi Arabia (KSA) between the years 2016 to 2017. One sample (GenBank ID OQ423284) clustered close to the MERS-CoV detected in the Al Hasa region (GenBank ID: MN654975.1), which was reported in 2016, while other isolates clustered close to the MERS-CoV isolate camel/KFU-HKU-I/2017 (GenBank: MN758606.1), which was reported in 2017.

The clustering of MERS-CoV sequences identified in this study was closely related to camel isolates, which may suggest camel-to-camel transmission and supports camels as an animal reservoir for

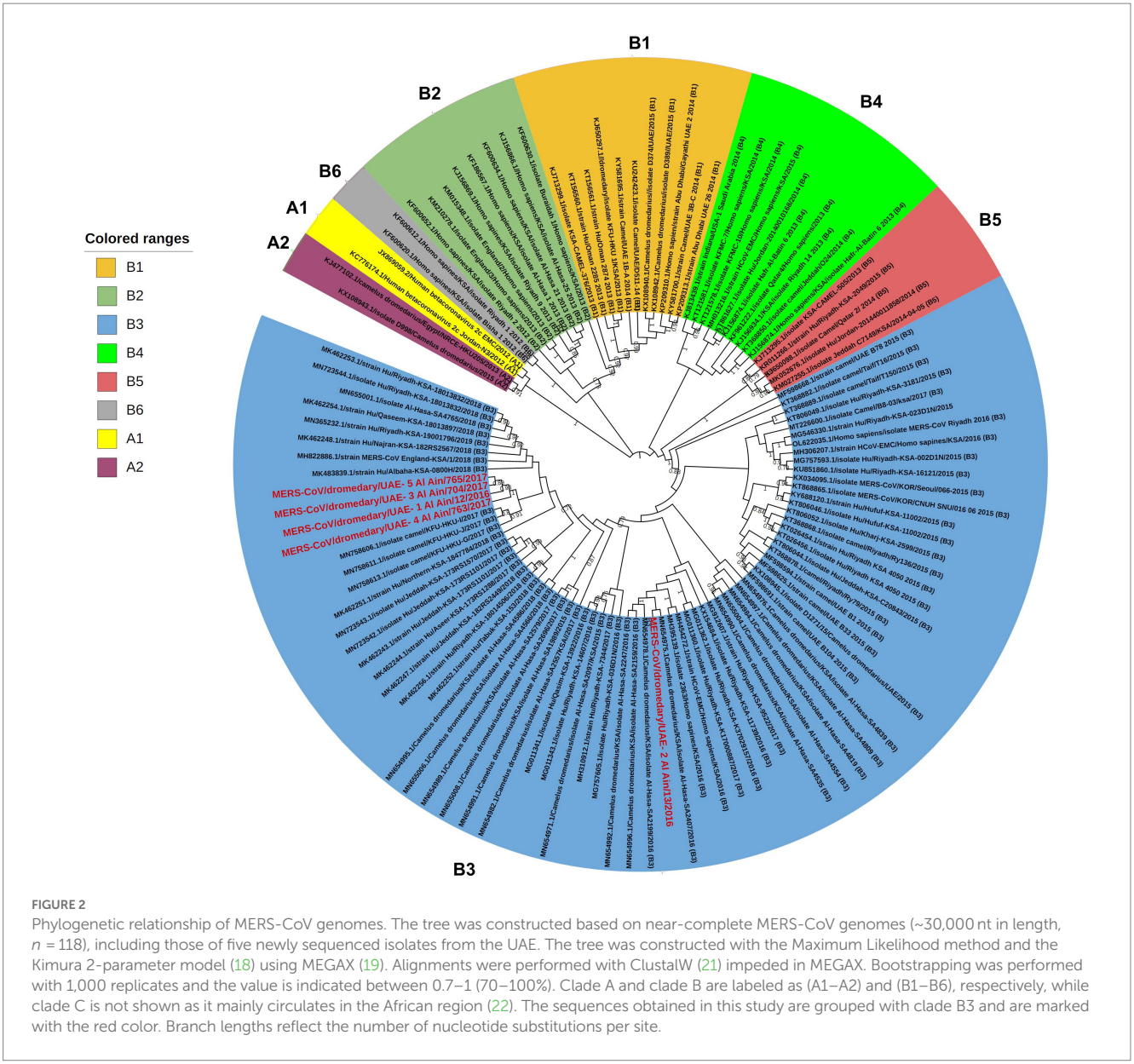

FIGURE 2
Phylogenetic relationship of MERS-CoV genomes. The tree was constructed based on near-complete MERS-CoV genomes (~30,000 nt in length, $n = 118$), including those of five newly sequenced isolates from the UAE. The tree was constructed with the Maximum Likelihood method and the Kimura 2-parameter model (18) using MEGAX (19). Alignments were performed with ClustalW (21) impeded in MEGAX. Bootstrapping was performed with 1,000 replicates and the value is indicated between 0.7–1 (70–100%). Clade A and clade B are labeled as (A1–A2) and (B1–B6), respectively, while clade C is not shown as it mainly circulates in the African region (22). The sequences obtained in this study are grouped with clade B3 and are marked with the red color. Branch lengths reflect the number of nucleotide substitutions per site.

MERS-CoV. Of note, the sequences of the current isolates were found to cluster distantly in the phylogenetic tree far from the previously known MERS-CoV isolated from camels in the UAE, which were located in lineage 5 and lineage 7 within clade B (8). Therefore, this study may provide insight into the risk of trades and livestock markets in the spread of MERS-CoV between camels.

In many studies, a high frequency of mutations and recombinant events were observed in MERS-CoV camel isolates (30) as well as human isolates, and this is necessary for the virus fitness and adaptation in the host.

In our study, where the virus was sequenced from cell culture-adapted isolates, several mutations were observed. For instance, 32 amino acid substitutions in the ORF1a, 25 amino acid substitutions in the ORF1ab, and a single substitution (p.Gly198Ser) in the N region were reported in samples with GenBank IDs: OQ423285 and OQ423287. The latter mutation was also previously reported in human isolates in KSA (31). Generally, amino acid substitutions in the nsp proteins, as well as the N protein, were found to reduce interaction and subsequent virus replication and progeny production (32).

Moreover, two missense amino acid substitutions in the S protein (p.Phe473Ser in sample 2 and p.Gln1020Arg in samples 2 and 3) were reported. It is known that the p.Phe473Ser is located in the receptor-binding domain (RBD) of the spike gene, and exclusively detected in camel strains in Jeddah and Riyadh (22, 33). The mutation p.Gln1020Arg (Q1020R) is located in the heptad repeat region (HR1) of the spike protein. Other mutations detected in our isolates such as Q1020R, R1020Q, or Q1020H were also reported in camel strains in Egypt, Nigeria, the UAE, and some KSA human strains in Jeddah (34–36); these were found to be associated with major MERS-CoV outbreaks in these countries (22). Therefore, changes in the coronavirus spike should be carefully monitored for their effects on receptor binding, virus transmission (37), or even interspecies jump and spillover to humans (31).

This study also identified three mutations in ORF4b in all isolates and one mutation in samples with GenBank IDs OQ423284 and

TABLE 2 The percent identity matrix created by ClustalW for the MERS-CoV isolates along with close sequence obtained after BLAST analysis.

Strain	OQ423283	OQ423284	OQ423285	OQ423286	OQ423287	MN758606.1	MN654975.1	JX869059
OQ423283/MERS-CoV/dromedary/UAE-Al Ain/12/2016		99.70%	99.98%	99.96%	99.99%	99.90%	99.74%	99.43%
OQ423284/MERS-CoV/dromedary/UAE-Al Ain /13/2016	99.70%		99.69%	99.70%	99.70%	99.73%	99.86%	99.47%
OQ423285/MERS-CoV/dromedary/UAE-Al Ain /704/2017	99.98%	99.69%		99.95%	99.99%	99.90%	99.74%	99.42%
OQ423286/MERS-CoV/dromedary/UAE-Al Ain /763/2017	99.96%	99.70%	99.95%		99.96%	99.91%	99.74%	99.42%
OQ423287/MERS-CoV/dromedary/UAE-Al Ain/765/2017	99.99%	99.70%	99.99%	99.96%		99.91%	99.75%	99.43%
MN758606.1/isolate camel/KFU-HKU-I/2017	99.90%	99.73%	99.90%	99.91%	99.91%		99.78%	99.46%
MN654975.1/dromedary/KSA/isolate Al-Hasa-SA2407/2016	99.74%	99.86%	99.74%	99.74%	99.75%	99.78%		99.49%
JX869059/Human betacoronavirus 2c EMC/2012	99.43%	99.47%	99.42%	99.42%	99.43%	99.46%	99.49%	

The MERS-CoV reference sequence (JX869059) was also included.

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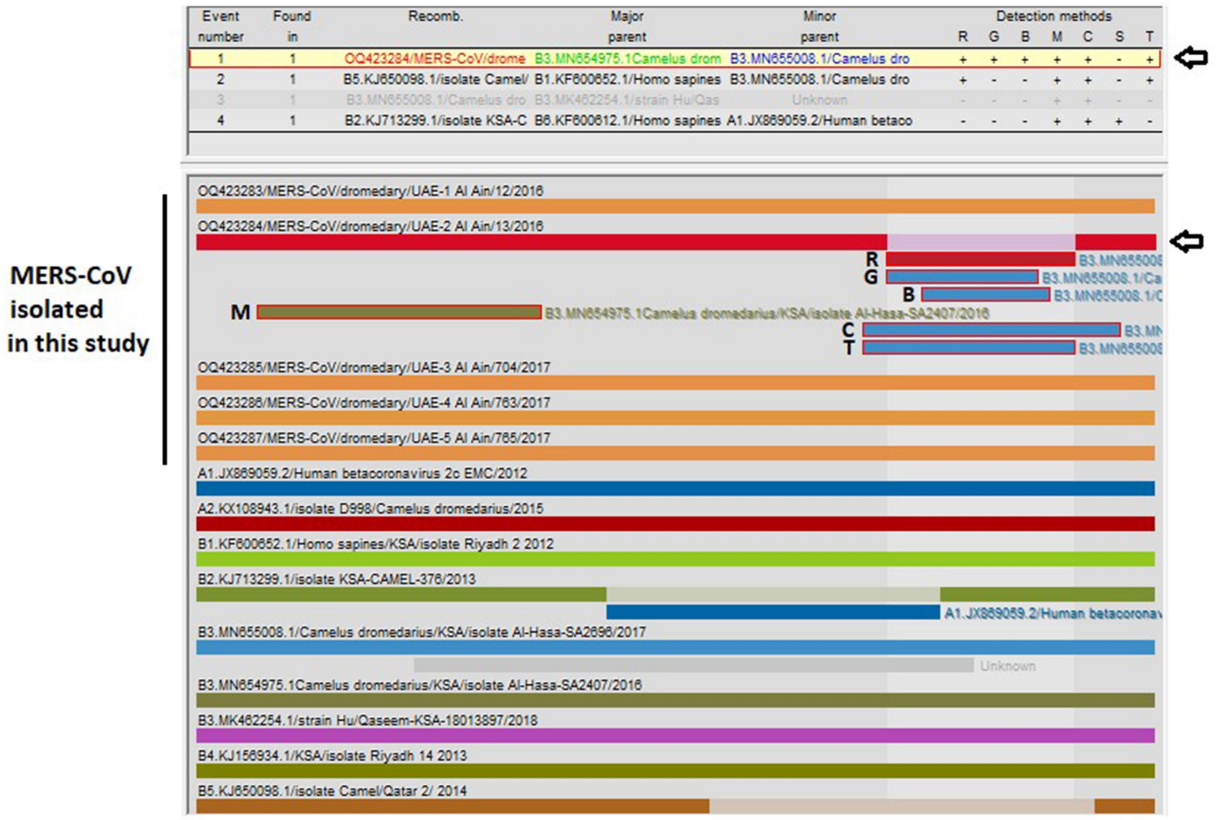

FIGURE 3
Analysis of possible recombination events in a nearly complete MERS-CoV genome (~30,000 bp). A single recombination event was detected in OQ423284/MERS-CoV/dromedary/UAE-2/Al Ain/13/2016 (indicated by a black arrow). The event detected by the six programs impeded in RDP5 program v.5.3 are indicated (R, RDP; G, GENECONV; B, BootScan; M, MaxiChi; C, Chimaera; T, 3Seq). Detailed information for recombination was also provided in Table 4. Each segment is indicated by a different color bar.

OQ423287 only. Amino acid substitutions in the ORF4b protein were found to alter the host's immune response to disease and viral pathogenesis (35, 38).

In addition, one mutation in ORF3 (p.Pro86Leu) observed in all current isolates was also reported previously in MERS-CoV strains in camels from the UAE (39). The biological impact of such amino acid changes in ORF3 (4), M (3), ORF8b (1), and among MERS-CoV strains needs to be fully examined.

Analysis of recombination indicated one of the reported sequences (OQ423284/MERS-CoV/dromedary/UAE-Al Ain/13/2016) is a recombinant strain containing sequence from Al Hasa strains (GenBank ID: MN654975.1 and GenBank ID: MN655008.1) within lineage 3 (Clade B3) with high similarity (99.9%). This is not surprising as recombination frequency due to the exchange of functional motifs or even entire genes is quite high (40) within closely related coronaviruses and between different coronaviruses (3, 41, 42). Recombination events generally affect the evolution and transmission of CoVs, including MERS-CoV, so positive selection sites in the spike protein of MERS-CoV in camels may have enabled virus spillover to humans (6, 26, 43). Therefore, the existence of a recombinant virus in our isolates implies the necessity of continuous surveillance of MERS-CoV infections and variants of concern in camels as well as humans, coupled with efforts toward the development of a MERS vaccine.

Artificial mutations could probably develop during cell culture passage even though the five MERS-CoV isolates analyzed in the current study were from early Vero cell passages and were still regarded as wild-type viruses. The fact that cell culture adaptation causes numerous artificial mutations when SARS-CoV-2 is isolated and multiplied in Vero-related cells is well documented (44). The recovery of MERS-CoV from synthesized RNA via the Baric approach in Vero cells is known to easily cause the T1015N artificial mutation in the S protein (45). Additionally, the repetitive passage of MERS-CoV on BHK cells that express DPP4 results in tailored mutations in the spike protein, even if Vero cells are not involved (46). Accordingly, the mutations described in this investigation might not accurately represent the virus's natural evolution. It is recommended that directly sequencing the wild-type virus from the positive samples using the amplicon method (36) or the SISPA method (16) is the next step to validate mutations reported in this study.

5. Conclusion

In conclusion, MERS-CoV genomic sequences determined in this study are similar to those of viruses detected in camels in Saudi Arabia during the period 2016–2018. Phylogenetic analysis of MERS-CoV isolates indicated the virus lineage 3 clade B viruses continue to be dominant among camels in the UAE. Sequence analysis identified several mutations in different virus proteins as well as a recombination event, raising concerns regarding new viral outbreaks and disease

TABLE 3 The missense variants identified from the five isolates (GenBank OQ423283 to OQ423287).

Position in the genome in the reference (JX869059.1)					nt	aa	Gene	Product
OQ423283	OQ423284	OQ423285	OQ423286	OQ423287				
301	301	301	301	301	c.23C > T	**p.Thr8Ile**	orf1ab	ORF1a
750	750	750	750	750	c.472 T > G	**p.Phe158Val**		
1833	1833	1833	1833	1833	c.1555C > A	**p.Leu519Ile**		
2040	2040	2040	2040	2040	c.1762G > A	**p.Ala588Thr**		
2,169	2,169	2,169	2,169	2,169	c.1891A > C	**p.Ile631Leu**		
X	2,456	2,456	X	X	c.2178A > C	p.Lys726Asn		
2,461	2,461	2,461	2,461	2,461	c.2183 T > G	**p.Ile728Ser**		
X	2,938	X	X	X	c.2660C > T	p.Thr887Ile		
X	3,397	X	X	X	c.3119C > T	p.Ala1040Val		
X	3,412	X	X	X	c.3134C > T	p.Ala1045Val		
3,441	3,441	3,441	3,441	3,441	c.3163C > T	**p.Pro1055Ser**		
3,487	3,487	3,487	3,487	3,487	c.3209C > A	**p.Ala1070Glu**		
3,559	3,559	3,559	3,559	3,559	c.3281A > G	**p.Asn1094Ser**		
3,574	X	3,574	3,574	3,574	c.3296C > G	p.Pro1099Arg		
X	3,882	X	X	X	c.3604G > A	p.Val1202Ile		
3,984	3,984	3,984	3,984	3,984	c.3706G > A	**p.Ala1236Thr**		
4,388	4,196	4,388	4,388	4,388	c.4110G > T	**p.Met1370Ile**		
4,401	4,388	4,401	4,401	4,401	c.4123G > A	**p.Val1375Ile**		
4,662	X	4,662	4,662	4,662	c.4384G > A	p.Ala1462Thr		
X	5,139	X	X	X	c.4861C > T	p.His1621Tyr		
5,427	5,427	5,427	5,427	5,427	c.5149 T > A	**p.Leu1717Ile**		
X	5,726	X	X	X	c.5448G > T	p.Glu1816Asp		
X	5,782	X	X	X	c.5504A > C	p.Glu1835Ala		
6,189	X	6,189	X	6,189	c.5911C > T	p.Pro1971Ser		
6,286	6,286	6,286	X	6,286	c.6008C > T	p.Ala2003Val		
6,619	6,619	6,619	6,619	6,619	c.6341C > T	**p.Ala2114Val**		
X	6,635	X	X	X	c.6357G > T	p.Met2119Ile		
X	7,555	X	X	X	c.7277C > T	p.Thr2426Ile		
8,518	8,518	8,518	8,518	8,518	c.8240C > T	**p.Ala2747Val**		
8,617	8,617	8,617	8,617	8,617	c.8339C > T	**p.Ala2780Val**		
9,516	9,516	9,516	9,516	9,516	c.9238A > G	**p.Thr3080Ala**		
13,396	13,396	13,396	13,396	13,396	c.13118C > T	**p.Ala4373Val**		

(Continued)

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TABLE 3 (Continued)

Position in the genome in the reference (JX869059.1)					nt	aa	Gene	Product
OQ423283	OQ423284	OQ423285	OQ423286	OQ423287				
13,678	13,678	13,678	13,678	13,678	c.13400C > T	**p.Thr4467Ile**		
14,992	14,992	14,992	14,992	14,992	c.14714 T > C	**p.Phe4905Ser**		
15,196	15,196	15,196	15,196	15,196	c.14918C > T	**p.Thr4973Met**		
15,835	15,835	15,835	15,835	15,835	c.15557G > A	**p.Gly5186Asp**		
15,985	15,985	15,985	15,985	15,985	c.15707G > A	**p.Gly5236Asp**		
16,174	16,174	16,174	16,174	16,174	c.15896 T > C	**p.Ile5299Thr**		
16,597	16,597	16,597	16,597	16,597	c.16319C > T	**p.Ser5440Leu**		
16,804	16,804	16,804	16,804	16,804	c.16526 T > C	**p.Phe5509Ser**		
17,089	X	17,089	17,089	17,089	c.16811G > A	p.Gly5604Asp		
17,752	17,752	17,752	17,752	17,752	c.17474C > T	**p.Thr5825Ile**		
17,771	17,771	17,771	17,771	17,771	c.17493 T > G	**p.Cys5831Trp**		
17,794	X	17,794	17,794	17,794	c.17516C > T	p.Pro5839Leu		
17,836	17,836	17,836	17,836	17,836	c.17558C > T	**p.Thr5853Met**	orf1ab	ORF1ab
18,079	18,079	18,079	18,079	18,079	c.17801 T > C	**p.Met5934Thr**		
18,112	X	18,112	18,112	18,112	c.17834 T > C	p.Met5945Thr		
18,146	18,146	18,146	X	18,146	c.17868G > A	p.Met5956Ile		
18,415	18,415	18,415	18,415	18,415	c.18137A > C	**p.His6046Pro**		
19,075	19,075	19,075	19,075	19,075	c.18797G > A	**p.Gly6266Glu**		
19,204	X	19,204	19,204	19,204	c.18926G > A	p.Cys6309Tyr		
19,940	19,940	19,940	19,940	19,940	c.19662 T > G	**p.Ile6554Met**		
19,999	19,999	19,999	19,999	19,999	c.19721C > T	**p.Ser6574Leu**		
20,017	20,017	20,017	20,017	20,017	c.19739G > T	**p.Trp6580Leu**		
20,182	20,182	20,182	20,182	20,182	c.19904 T > G	**p.Phe6635Cys**		
20,848	20,848	20,848	20,848	X	c.20570C > A	p.Pro6857His		
X	X	X	X	20,848	c.20570C > A	p.Pro6857His		
X	22,873	X	X	X	c.1418 T > C	p.Phe473Ser	S	S protein
X	24,514	24,514	X	X	c.3059A > G	p.Gln1020Arg		

(Continued)

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TABLE 3 (Continued)

Position in the genome in the reference (JX869059.1)					nt	aa	Gene	Product
OQ423283	OQ423284	OQ423285	OQ423286	OQ423287				
25,580	25,580	X	25,580	25,580	c.49C > T	p.Leu17Phe	orf3	ORF3
25,715	25,715	25,715	25,715	25,715	c.184G > T	**p.Val62Phe**		
25,761	25,761	25,761	25,761	25,761	c.230 T > G	**p.Leu77Arg**		
25,788	25,788	25,788	25,788	25,788	c.257C > T	**p.Pro86Leu**		
26,109	26,109	26,109	26,109	26,109	c.17 T > C	**p.Met6Thr**	orf4b	ORF4b
26,167	26,167	26,167	26,167	26,167	c.316C > T	**p.Pro106Ser**		
26,223	26,223	26,223	26,223	26,223	c.131A > C	**p.His44Pro**		
X	26,508	X	X	X	c.416C > T	p.Ala139Val		
28,057	28,057	28,057	28,057	28,057	c.205G > A	p.Val69Ile	M	M protein
X	28,102	X	X	X	c.250G > T	p.Ala84Ser		
28,219	28,219	28,219	28,219	28,219	c.367 T > A	**p.Phe123Ile**		
28,772	28,772	28,772	28,772	28,772	c.11 T > C	**p.Leu4Pro**	orf8b	ORF8b
X	X	X	29,157	X	c.592G > A	p.Gly198Ser	N	N protein

nt, nucleotide; aa, amino acid; and x denotes an absence. Missense variants in bold are shared between the five isolates (GenBank OQ423283 to OQ423287).

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TABLE 4 Summary of the recombination event identified by the Recombination Detection Program (RDP5) v.5.3.

Recombinant	OQ423284/MERS-CoV/dromedary/UAE-Al Ain /13/2016	No of sequence detected	Beginning breakpoint (position in the alignment)	Ending breakpoint (position in the alignment)	Av. *p*-value
Major parent	B3.MN654975.1/*Camelus dromedarius*/KSA/isolate Al-Hasa-SA2407//2016				
Minor parent	B3.MN655008.1/*Camelus dromedarius*/KSA/isolate Al-Hasa-SA2696/2017				
p-value determined by six different algorithms	RDP	1	20,857 (99.9% similarity)	27,414 (99.9% similarity)	1.271×10^{04}
	GENECONV	1	21,588 (99.9% similarity)	26,276 (100% similarity)	1.011×10^{03}
	BootScan	1	22,672 (99.9% similarity)	26,635 (99.9% similarity)	1.304×10^{02}
	MaxiChi	1	1930 (99.8% similarity)	10,772 (100% similarity)	1.593×10^{04}
	Chimaera	1	20,857 (99.9% similarity)	28,866 (99.9% similarity)	1.140×10^{03}
	3Seq	1	20,857 (99.9% similarity)	27,414 (99.9% similarity)	3.834×10^{07}

severity. The results of this study support the necessity for the complete genome sequencing of all new cases of MERS-CoV to monitor virus circulation in the region and to develop effective control measures. Although the mutations detected in this study may not reflect the true natural evolution of the virus, as artificial mutations may occur during cell culture passage, the isolated MERS-CoV would be useful in new vaccine development and efficacy studies, pathogenicity, and antiviral research.

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 current study involving swab samples from dromedary camels was reviewed and approved by the ADAFSA Ethics Committee for Monitoring the use of Animals in Scientific Research (ADAFSA-EA-08-2016) and the use of these samples for virus isolation was permitted without additional ethical approval. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AK and HI: conceptualization and data analysis. AK, HI, and SM: methodology, data curation, and writing-original draft preparation. HA and ZA-H: sample preparation and testing. AK, HI, and AS: writing-review and editing. AS and SM: supervision and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1182165/full#supplementary-material

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Zoonotic diseases transmitted from the camels

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Background: Zoonotic diseases, infections transmitted naturally from animals to humans, pose a significant public health challenge worldwide. After MERS-CoV was discovered, interest in camels was raised as potential intermediate hosts for zoonotic viruses. Most published review studies pay little attention to case reports or zoonotic epidemics where there is epidemiological proof of transmission from camels to humans. Accordingly, any pathogen found in camels known to cause zoonotic disease in other animals or humans is reported.

Methods: Here, zoonotic diseases linked to camels are reviewed in the literature, focusing on those with epidemiological or molecular evidence of spreading from camels to humans. This review examines the risks posed by camel diseases to human health, emphasizing the need for knowledge and awareness in mitigating these risks.

Results: A search of the literature revealed that eight (36.4%) of the 22 investigations that offered convincing evidence of camel-to-human transmission involved MERS, five (22.7%) Brucellosis, four (18.2%) plague caused by *Yersinia pestis*, three (13.6%) camelpox, one (4.5%) hepatitis E, and one (4.5%) anthrax. The reporting of these zoonotic diseases has been steadily increasing, with the most recent period, from 2010 to the present, accounting for 59% of the reports. Additionally, camels have been associated with several other zoonotic diseases, including toxoplasmosis, Rift Valley fever, TB, Crimean-Congo hemorrhagic fever, and Q fever, despite having no evidence of a transmission event. Transmission of human zoonotic diseases primarily occurs through camel milk, meat, and direct or indirect contact with camels. The above-mentioned diseases were discussed to determine risks to human health.

Conclusion: MERS, Brucellosis, plague caused by *Y. pestis*, camelpox, hepatitis E, and anthrax are the main zoonotic diseases associated with human disease events or outbreaks. Transmission to humans primarily occurs through camel milk, meat, and direct contact with camels. There is a need for comprehensive surveillance, preventive measures, and public health interventions based on a one-health approach to mitigate the risks of zoonotic infections linked to camels.

KEYWORDS

camels, zoonotic diseases, camel-to-human transmission events, literature review, risks to human health

1. Introduction

Zoonosis refers to the transmission of diseases or infections from vertebrate animals to humans in a natural manner (1). These incidences pose a significant challenge to public health on a global scale, primarily due to our intimate connection with animals in various contexts such as agriculture, companionship, and the natural world. Multiple sources have claimed that at least 60% of emerging infectious diseases (EIDs) affecting humans are naturally zoonotic, originating from animal hosts other than humans. Moreover, zoonotic pathogens have a twofold higher likelihood of being connected to the emergence of diseases than non-zoonotic pathogens (2–4). Climate change, land use change, agricultural practices, animal movements in search of food and water, international trade in livestock and their products, international travel and tourism, and pathogen evolution are all factors that have greatly influenced the emergence of zoonotic pathogens (5).

There are mainly two species of old-world camels: *Camelus dromedarius* (dromedary, Arabian, or one-humped camel), found in the Middle East, North, and Northern East Africa, and other parts of the world, and *Camelus bactrianus* (Bactrian, or two-humped camel), which is primarily found in northwestern China and southwestern Mongolia. Dromedaries have a long-standing association with human societies, particularly in the Middle East, North, and Northeast Africa. Their unique physiological adaptations, such as efficient water economy mechanisms and heat tolerance, enable them to survive in arid environments (6).

Over the past decade, the global camel population has experienced significant growth, with a particular increase in dromedaries (7, 8). This expansion, combined with socio-cultural practices, intensification of production, and concentration of camel farming, has increased the risk of zoonotic diseases associated with camels.

Throughout the dromedary camel belt from Mauritania to India, socio-cultural practices combined with weak public and animal health infrastructures, the recent intensification of the production, the concentration of the production around cities, and the change to camel farming favor the occurrence and spread of zoonosis. In recent years, camel production has been steady growth/expanding both in terms of intensification, such as the appearance of large dairy farms mainly in the Arabian Gulf, which represent a sizable growing business, or geographically as in Africa. For instance, the Borana communities of Kenya, traditionally known as cattle herders, have switched their preference from raising cattle to camels, as camels represent a viable option for these drought-affected ecosystems (7, 9). Furthermore, according to Gossner et al. (10), camel production has intensified in the Arabian Peninsula, making it easier for zoonotic diseases to "spill over" from camels to people. This would explain how the Middle East respiratory syndrome coronavirus (MERS-CoV) first appeared in the human population of the Arabian Peninsula.

The emergence of MERS-CoV infection in 2012 has attracted interest in camel diseases that can be transmitted to humans, and accordingly, several pathogens have been reported in the literature utilizing recent advances in molecular diagnostic techniques. The conclusion is that there is a risk to the public's health since camels can contract many zoonotic infections. This ability of camels to be a potential source of diseases is a significant concern because more people consume their meat and milk. Therefore, knowledge of camel-associated zoonotic diseases and determining camels' risks to human health are essential.

2. Current knowledge of zoonotic diseases associated with camels

Recent publications have focused on identifying camel pathogens without emphasizing diseases conclusively transmitted from camels to humans. Sazmand et al. (11) reviewed zoonotic parasites in dromedary camels. They identified 13 zoonotic parasites in camels, including *Trypanosoma species* (spp.), *Giardia duodenalis*, *Enterocytozoon* spp., *Balantidium coli*, *Toxoplasma gondii*, *Cryptosporidium* spp., *Blastocystis* spp., *Fasciola* spp., *Schistosoma* spp., *Echinococcus granulosus*, *Trichinella* spp., *Sarcoptes scabiei* var. *cameli*, and *Linguatula serrata*. However, to my knowledge, none of these parasitic pathogens have been reported as causing human diseases through camel transmission.

Similarly, Mohammadpour et al. (12) reviewed the zoonotic implications of camel diseases in Iran. They identified 19 important zoonotic diseases reported in Iranian camels, including 11 bacterial, four viral, and four parasitic diseases. However, the authors only considered the incidence of camel diseases caused by pathogens known to be zoonotic without addressing the actual transmission of these diseases from camels to humans.

In their review of camel-associated zoonoses in Kenya, Hughes and Anderson (13) mentioned zoonotic infections found in camels, similar to the studies discussed above. Among the 16 pathogens identified were *Trypanosoma* spp., *E. granulosus*, *Brucella* spp., MERS-CoV, Rift Valley Fever virus, *Coxiella burnetii*, CCHF, *Dermatophilus congolensis*, and contagious ecthyma virus. According to Zhu et al. (14), Brucellosis, the Middle East Respiratory Syndrome Coronavirus (MERS-CoV), *E. granulosus*, and Rift Valley Fever (RVF) were the subjects of the majority of papers on zoonotic camel diseases (65%).

Therefore, compiling the most recent scientific data on camels' direct contribution to the spread of zoonotic illnesses is necessary.

3. Searching for zoonotic diseases that camels have been shown to transmit to humans

Relevant publications on zoonoses associated with camels were searched using several search terms, including "zoonoses camels, diseases transmitted by camels, as well as specific disease terms such as MERS, *Yersinia pestis* in camels, rabies in camels, camelpox in humans/people, human brucellosis from camels." The search was confined to authentic resources from repositories of popular databases: PubMed, Google Scholar, and SCOPUS.

This document only included compelling proof of camel-to-human transmission. All non-verified sources of information and studies describing the serological detection of human pathogens in camels were excluded from this review. Human zoonotic incidents in the dataset that have been identified as possibly involving camels are included in this study if (1) there is proven epidemiological data that the patient gets the infection from camels or their products or (2) sequence analysis of the causative agent from the patient and the camel samples revealed a close homology. For instance, this review

does not include rabies, though it is the most important zoonotic disease worldwide. The reason is that no disease event confirmed the association of camels with human rabies.

After investigating rabies in camels in the Qassim region of central Saudi Arabia, Al-Dubaib (15) also came to the conclusion that camels do not transmit rabies to humans.

When evaluating the outcomes of infectious diseases associated with farm animals, one will find that diseases like MERS-CoV infection with a case fatality rate in humans of roughly 34% and that plaque caused by *Y. pestis* are predominantly disseminated from camels rather than cattle or small ruminants.

4. Major zoonotic diseases directly associated with the camels

Using the critical search terms, 872 scientific reports and articles were identified, 22 of which satisfied the inclusion criteria and were analyzed. Eight (36.4%) of the 22 publications examined dealt with MERS, five (22.7%) with Brucellosis, four (18.2%) with plague caused by *Y. pestis*, three (13.6%) with camelpox, one (4.5%) with hepatitis E, and one (4.5%) with anthrax (Table 1 and Figure 1).

While some identified diseases, like MERS-CoV and HEV infections, do not manifest in the camel but can still seriously affect humans, others, like camelpox and brucellosis, infect both hosts. The first documented zoonotic disease associated with camels, a case of Plague (*Y. pestis* infection), was reported in Libya in February 1976 (30), while the most recent case was an outbreak of MERS-CoV in 2018 and 2020 (24). Figure 2 shows a constant increase in reporting these zoonotic diseases from 1980 to 2022, with the most recent period, from 2010 to the present, accounting for 59% of the reports.

Consumption of camel milk, meat, or direct or indirect contact with camels is an essential source of human zoonotic disease transmission (Figure 3). The countries where the transmission of zoonotic diseases from dromedary camels to humans has been confirmed are shown in Figure 4. Zoonotic diseases are displayed on the map by country, and the year they first emerged. These countries are located in the camel belt and include seven Asian and six African countries, extending from Libya in the west to India in the east.

TABLE 1 Details of camel-associated zoonotic diseases.

Disease	Country	Date	The zoonotic transmission proved by	Reference
MERS-CoV	Qatar	October 2013	Sequence analysis	Haagmans et al. (16)
MERS-CoV	Saudi Arabia	November 2013	Epidemiologic link and sequence analysis	Azhar et al. (17, 18)
MERS-CoV	Saudi Arabia	November 2013	Sequence analysis	Memish et al. (19)
MERS-CoV	UAE	July 2013–May 2014	Epidemiologic link and sequence analysis	Paden et al. (20)
MERS-CoV	UAE	February–May 2014	Sequence analysis	Al Muhairi et al. (21)
MERS-CoV	Saudi Arabia	2014–2016	Sequence analysis	Kasem et al. (22)
MERS-CoV	UAE	May 2015	Epidemiologic link and sequence analysis	Al Hammadi et al. (23)
MERS-CoV	Kenya	April 2018–March 2020	Sequence analysis	Ngere et al. (24)
Brucellosis	UAE	2008	Epidemiologic link and sequence analysis	Schulze zur Wiesch et al. (25)
Brucellosis	Israel	June 2011	Epidemiologic link and lab analysis	Shimol et al. (26)
Brucellosis	Israel	July–November 2016	Sequence analysis	Bardenstein et al. (27)
Brucellosis	Somalia, Ethiopia, Djibouti	2007 and 2013	Epidemiologic link	Rhodes et al. (28)
Brucellosis	Qatar	February 2015	Epidemiologic link	Garcell et al. (29)
Plague (*Y. pestis* infection)	Libya	February 1976	Epidemiologic link	Christie et al. (30)
Plague (*Y. pestis* infection)	Saudi Arabia	February 1994	Epidemiologic link	Bin Saeed et al. (31)
Plague (*Y. pestis* infection)	Afghanistan	December 2007	Epidemiologic link	Leslie et al. (32)
Plague (*Y. pestis* infection)	Jordan	February 1997	Epidemiologic link	Arbaji et al. (33)
Camel pox	Somalia	1987	Epidemiologic link	Kriz (34)
Camel pox	India	April–May 2009	Epidemiologic link	Bera et al. (35)
Camel pox	Sudan	September–December 2014	Epidemiologic link	Khalafalla and Abdelazim (36)
Hepatitis E infection	UAE	July 2012	Epidemiologic link and sequence analysis	Lee et al. (37)
Anthrax	Sudan	February 1988	Epidemiologic link	Musa et al. (38)

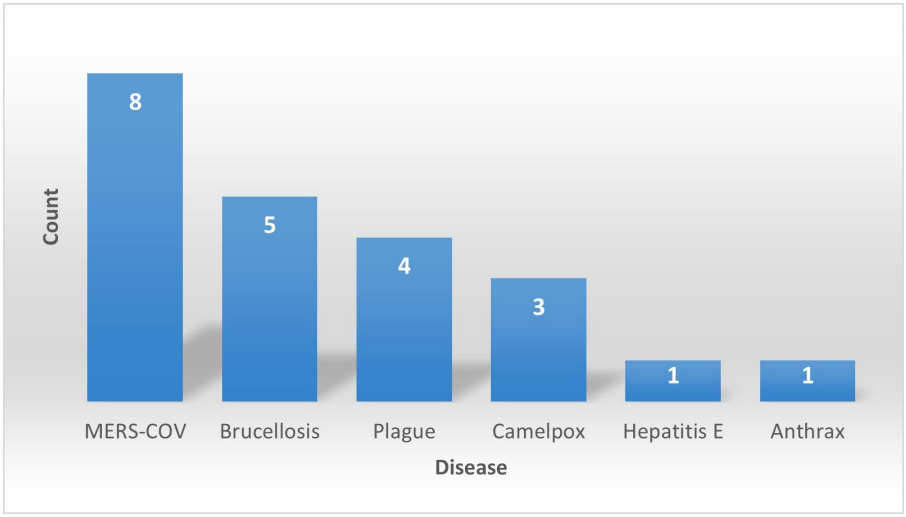

FIGURE 1
Camel diseases and records of human zoonotic occurrences.

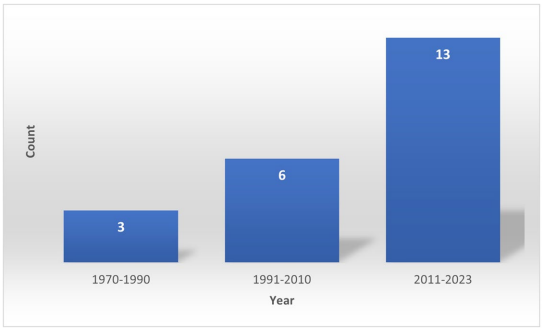

FIGURE 2
Distribution of records on camel zoonotic disease events by year of occurrence.

4.1. MERS-CoV infection

Middle East Respiratory Syndrome (MERS) is a viral respiratory system disease caused by the MERS coronavirus (MERS-CoV), which first emerged in Saudi Arabia in 2012. Structured coalescent models demonstrated that long-term MERS-CoV evolution only occurs in camels. The seasonally variable zoonotic transmission of viruses from camels is the primary cause of human epidemics in the Arabian Peninsula (39). Dromedary camels appear to be the only animal host responsible for the spillover of human infections, although bats and alpacas can act as potential reservoirs for MERS-CoV (40). Phylogenetic and sequencing data strongly suggest that MERS-CoV likely originated from bat ancestors due to a recombination event in the spike protein, which may have occurred in dromedary camels in Africa before being transported to the Arabian Peninsula via camel trade routes (41). Two of the initial MERS-CoV cases in Qatar were men who attended Doha's main livestock market and the neighboring central slaughterhouse. According to Farag et al. (42), camel slaughterhouses are high-risk areas for human exposure where MERS-CoV is circulating. The research community responded quickly to the high fatality rates from MERS outbreaks between 2012 and 2016, as evidenced by the abrupt rise in MERS-related publications.

Eight publications provided convincing evidence of camel-to-human transmission; sequencing comparisons between the patient and camel samples showed high similarity. The studies employ various methodologies, including virus isolation, sequencing, and epidemiological investigations, to establish a strong relationship between camels and human cases of MERS-CoV infection.

Azhar et al. (17) isolated and sequenced MERS-CoV from a dromedary camel and a patient with laboratory-confirmed MERS-CoV infection. The whole genome sequences of the two isolates were found to be identical. In a second study, the researchers found identical MERS-CoV RNA sequences in an air sample infected with the virus from the camel's barn (18). Memish et al. (19) analyzed viral sequences from humans and camels, revealing nucleotide polymorphism signatures indicative of cross-species transmission. A farm in Qatar was associated with two human cases of MERS-CoV infection, according to research conducted by Haagmans et al. (16). The authors detected the virus in nasal swabs from three camels. The nucleotide sequences of the camel isolates were highly similar to those obtained from the infected humans on the same farm, indicating a potential outbreak affecting both species. Similarly, Al Hammadi et al. (23) identified asymptomatic MERS-CoV infection in two men exposed to infected dromedaries in the United Arab Emirates. The genetic sequences of MERS-CoV from the men and camels exhibited similarities with those detected in other countries, supporting zoonotic transmission. Al Muhairi et al. (21) conducted a comprehensive study involving 1,113 dromedary camels and two MERS-CoV-infected camel farm owners in the UAE. Sequencing analysis revealed that the camel sequences and sequences from one farm owner clustered within the larger MERS-CoV sequence cluster, further demonstrating the zoonotic odds of MERS-CoV transmission.

Additional studies from Kasem et al. (22), Paden et al. (20), and Ngere et al. (24) provided further evidence of camel-to-human transmission. Kasem et al. (22) found complete genome sequence

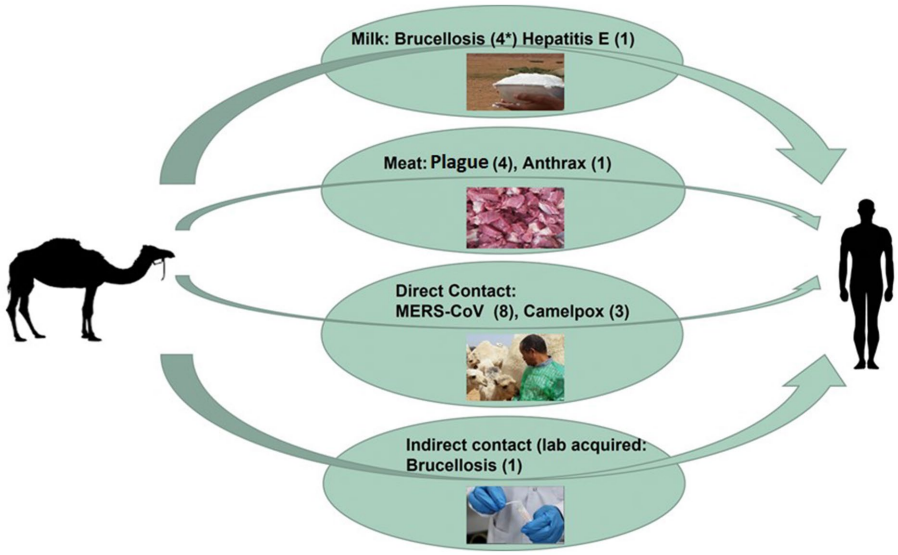

FIGURE 3
Overview diagram of camel-borne zoonotic disease transmission routes. The overall number of zoonotic disease incidents is displayed between two brackets (*).

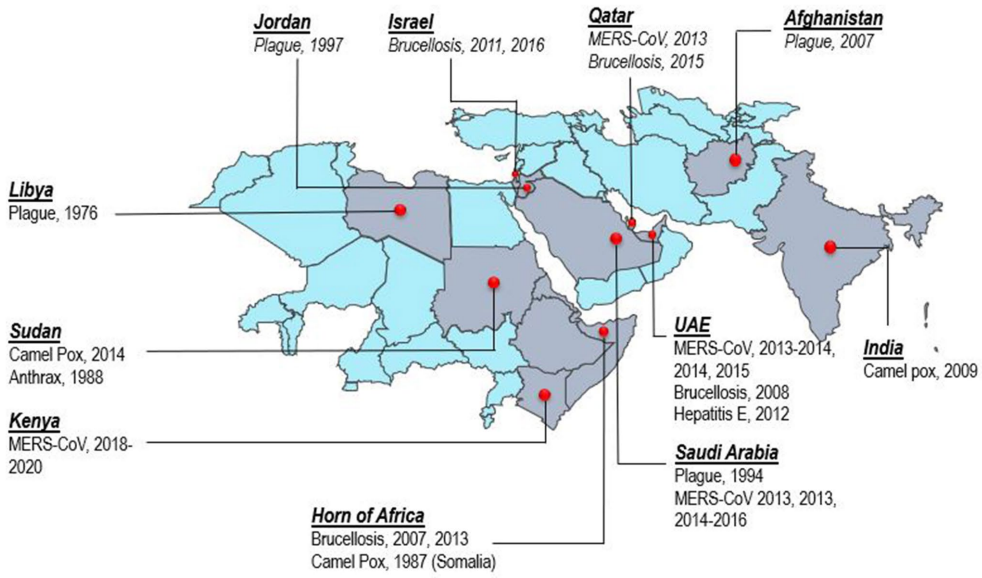

FIGURE 4
Map of camel belt displaying countries where zoonotic disease transmission from dromedary camels to humans has been documented. The map depicts zoonotic diseases per country with the year of reporting.

identity between MERS-CoV isolates. Paden et al. (20) sequenced whole MERS-CoV genomes from respiratory samples collected from human cases and camels in the UAE, finding nearly identical genomes between the two groups. Finally, Ngere et al. (24) demonstrated sporadic transmission of MERS-CoV from camels to humans during intense outbreaks in Kenya. As per the researchers, an analysis of camel swabs collected from calves in Kenya between April and September 2019 yielded interesting results. Of the 4,692 swabs collected from 83 calves in 15 homesteads, 2.6% (124 samples) tested positive for the virus. Additionally, it was observed that 22 calves (26.5%) showed reinfections, indicating a subsequent positive swab after at least two consecutive negative tests. Further investigation through sequencing unveiled the presence of a distinct clade C2 virus. Unlike other clade C viruses, this strain did not exhibit the typical ORF4b deletions. Notably, three previously reported cases of humans testing positive for the virus via PCR clustered temporally and geographically with the camel infections. These findings suggest that sporadic transmission from camels to humans occurred during the peak of camel outbreaks in Northern Kenya. Previous studies have established the widespread occurrence

of MERS-CoV in Kenyan camels, confirming its geographical presence (20, 43).

4.1.1. Current gaps and priorities for research

Based on epidemiological and virological studies, the dromedary camel is the most likely source of human MERS-CoV infections. According to some theories, MERS-CoV may have started in bats and then spread to camels (44–46). However, the ancestral origin of MERS-CoV and the exact source and mechanism of direct transmission to humans remain unknown. Comparatively, few publications of MERS-CoV genome analysis from humans and 8 camels have been published (46). Therefore, continuous surveillance and genomic investigations are required to investigate the virus's spread and evolution among camels and to find the variation of concerns (VOCs) linked with pandemic potential, as in the scenario with SARS-CoV-2. Furthermore, the identification of MERS-CoV in *Hyalomma dromedarii* ticks attached to dromedary camels in the United Arab Emirates (47) has raised intriguing issues about the potential role of arthropod vectors in the disease's transmission. Consequently, it would be interesting to determine if *H. dromedarii* is a competent vector for MERS-CoV, and its potential significance in mechanical or fomite transmission between camels should be explored. Additionally, research directed towards developing vaccines for camels to limit spillover infections to humans, validation of the current diagnostic techniques, and active surveillance of MERS-CoV in camels would be of great value in implementing sound mitigation interventions.

4.2. Brucellosis

The bacterial disease known as brucellosis, which can affect both humans and animals, is caused by many species of the genus *Brucella*. It is considered one of the most common neglected zoonotic diseases, primarily impacting the reproductive systems. In animals, infection with Brucella is characterized by signs such as abortion, infertility, retained placenta, orchitis, epididymitis, and, uncommonly, arthritis. The organisms can be excreted in uterine discharges, milk, urine, and semen (48).

Human brucellosis is mainly transmitted by consuming contaminated raw dairy products and meat from domestic animals. Except for Australia, camel brucellosis cases have been recorded in almost all countries raising camels. Some countries are experiencing an increase in the prevalence of the disease among camels due to unrestricted trade in live animals and inadequate measures to control its spread.

Evidence of disease transmission from camels to humans has been documented in five publications from the United Arab Emirates (UAE), the Horn of Africa, Qatar, and Israel. In a notable case in the UAE, Schulze zur Wiesch et al. (25) reported the diagnosis of acute brucellosis in a veterinarian working in a veterinary laboratory. The genomic analysis identified matching brucella strains in dromedary milk samples and patient cultures, supporting the diagnosis of laboratory-acquired infection. This study emphasized the high brucellosis infectivity, including airborne transmission, in veterinary laboratories handling camel specimens, highlighting the need for robust biosafety measures. Another study by Shimol et al. (26) described an outbreak of human brucellosis in Israel, where drinking camel milk was identified as the mode of infection. In this case, 15 patients were diagnosed with acute brucellosis, and *Brucella melitensis* was confirmed in their blood cultures and the camel's milk through serology and culture. Whole-genome sequencing in Israel linked patients with *B. melitensis* to uncontrolled livestock trading and wholesale camel milk (27).

Previously, reports implicating camel milk as a source of brucella infection were limited to patients residing in or traveling to and from the Middle East. However, Rhodes et al. (28) reported invasive human brucellosis infections in travelers and immigrants from the Horn of Africa (Somalia, Ethiopia, and Djibouti), highlighting the consumption of raw camel milk as the source of infection. Furthermore, Garcell et al. (29) reported an outbreak of human brucellosis in a rural area in Qatar, where 14 family members who owned camels and sheep were affected. The source of the infection was identified as the milk of an infected camel, which was confirmed through serological studies conducted on the patients.

These studies collectively contribute to the understanding of camel-associated brucellosis and affirm the importance of considering camels as potential sources of zoonotic diseases, mainly through the consumption of camel milk.

4.2.1. Current gaps and priorities for research

According to several researchers, breeding camelids experience fewer abortions due to brucella infection than cattle and small ruminants (49). Abu Damir et al. (50) experimentally infected camels with bacterial strains (S19 and a field bovine strain) in the only publication of its kind. It is, therefore, necessary to experimentally infect camels with camel-isolated *Brucella* strains to understand the pathogenesis, pathology, clinical symptoms, and bacterial shedding.

Future studies should focus on determining the pathology and pathogenesis of *Brucella* strains in experimentally infected camels, assessing the risk of zoonotic infection in laboratories, and evaluating immunization strategies. Abbas and Agab (51) proposed endeavoring vaccination of camels with *B. abortus* strain RB51 in this context. This strain was tried successfully in adult cattle and bison with many benefits, including a lack of interference with serological diagnosis. The authors also suggested bacteriologic surveys to conclude the relative importance of brucella species (*B. melitensis* and *B. abortus*) in the etiology of camel brucellosis.

4.3. Camel plague (*Yersinia pestis* infection)

Y. pestis, an anaerobic bacterium, is responsible for causing the human plague or the Black Death, a fatal disease spread by flea bites from naturally infected rats to people. However, camels and other mammals can contract the disease (52). During the middle-age, plague pandemics claimed the lives of approximately 200 million people. Although the intensity has significantly decreased, the natural foci of plague persist in many locations around the globe, particularly in Africa and Asia. Camelids (Dromedary and Bactrian camels, New World Camelids) are susceptible to *Y. pestis*, and plague cases have been reported in these animals in different regions where these animals are reared. In contrast to other agricultural animals, the wide-ranging behavior of camels enhances their tendency to come into contact with natural habitats of plague (33). Ancient Arabs believed

that the excessive death of camels was a warning of an approaching human plague because, in the past, many humans had contracted the disease from camels (52). From the former USSR, there have been several cases of *Y. pestis* spreading from plague-infected camels to people during the early years of the previous century.

Recently, four studies have indicated a connection between *Y. pestis* infection and contact with dromedary camels. In 1997, Arbaji et al. (33) investigated a plague outbreak in a Jordanian village marked by fever and cervical lymphadenopathy. The affected individuals reported consuming raw or cooked meat from the same camel's carcass. Bin Saeed et al. (31) examined a cluster of five plague cases in Saudi Arabia, including four individuals with severe pharyngitis and submandibular lymphadenitis in 1994. The four patients had consumed raw camel liver. *Y. pestis* was isolated from the camel's bone marrow, and fleas and jirds were caught in the camel pen. Leslie et al. (32) reported an outbreak of plague in Afghanistan with a rare gastrointestinal presentation associated with consuming or handling camel meat. Seventeen people died as a result of the outbreak. Molecular and immunological testing of patient clinical samples and camel tissue revealed DNA signatures consistent with *Y. pestis*. Christie et al. (30) described an incident in Libya in 1976 where the meat of a slaughtered sick camel was distributed for human consumption. Several days later, 15 villagers fell severely ill with a febrile illness, and those who had participated in the slaughtering and distribution of the camel all died within 4 days. Using the passive hemagglutination test, samples from the remaining patients showed evidence of plague.

In conclusion, evidence suggests that humans can contract *Y. pestis* from dromedary camels. Cases of plague associated with the consumption of camel meat or contact with infected camels have been reported in various regions. This highlights the importance of implementing appropriate preventive measures and raising awareness about the potential risks associated with camel-related zoonotic diseases.

4.3.1. Current gaps and priorities for research

Y. pestis is a major meat-borne zoonotic bacterial pathogen, and its management necessitates action at the point where people, animals, and their environments interact. Until recently, plague in camels was diagnosed only after the animal's death; such a diagnosis was not established in living camels (52). Therefore, research is needed to develop diagnostic tools for rapidly detecting and confirming *Y. pestis* before and post-mortem at slaughterhouses. Limited trials of the anti-plague vaccines in camels have been conducted, and genetically modified vaccines are also recently developed to protect both humans and animals from the plague (52). Vaccination trials in camels should be investigated to determine the best dose, safety, and efficacy. Furthermore, an assessment of risk factors in human and animal populations and the socioeconomic impacts of the disease are required.

4.4. Hepatitis E infection

The hepatitis E virus (HEV) is the primary cause of the emerging zoonotic enteric disease known as hepatitis E, and belongs to the *Orthohepevirus* genus in the family *Hepeviridae*. This family comprises four species: *Orthohepevirus A–D*. HEV is primarily transmitted through the fecal-oral route and is well-known as a zoonotic pathogen (53).

Woo et al. (54) reported a novel genotype of HEV identified in dromedaries, suggesting another potential source of human HEV infection. A molecular epidemiology study in Dubai, United Arab Emirates, detected HEV in fecal samples from three camels. Complete genome sequencing of two strains revealed more than 20% nucleotide difference compared to known HEVs. A previously unknown genotype, ultimately identified as HEV7, a novel *Orthohepevirus A* genotype exclusive to dromedaries, was discovered by comparative genomic and phylogenetic analysis (55, 56).

A new HEV genotype was detected in Bactrian camels in 2016 in Xinjiang, China. Sequence analysis demonstrated that the three Bactrian HEV strains represented a distinct genotype, currently classified as HEV8 (57). Despite successfully identifying genotypes 7 and 8 in dromedaries and Bactrian camels, these viruses' epidemiology, zoonotic potential, and pathogenicity remained unclear.

Only one study has provided evidence of virus transmission from camels to humans. Lee et al. (37) conducted partial and full-length phylogenetic analyses of HEV sequences from a liver transplant patient in the Middle East and compared them with other *Orthohepevirus A* sequences. According to the findings, people who routinely consume camel milk and meat were infected with camelid HEV. The authors concluded that camelid HEV, specifically genotype 7, might be capable of infecting humans.

In conclusion, hepatitis E is an emerging zoonotic disease. Novel genotypes (HEV7 and HEV8) have been discovered in dromedaries and Bactrian camels. While the zoonotic potential of these viruses is not yet fully understood, evidence suggests the transmission of camelid HEV (genotype 7) to humans through camel meat and milk consumption.

4.4.1. Current gaps and priorities for research

Limited knowledge exists regarding the amount of foodborne transmission and the role played by camels in the zoonotic spread of HEV to people. Research is needed to investigate these camel-associated HEV genotypes' epidemiology, zoonotic transmission dynamics, and pathogenicity.

4.5. Camelpox

Camelpox is a highly infectious skin disease and the most encountered viral infection of Old-World camelids (Dromedary and Bactrian camels), endemic in almost every country where camel husbandry is practiced, except for Australia (58). The camelpox virus (CMLV), a member of the *Orthopoxvirus* (OPXV) genus in the *Chordopoxvirinae* subfamily of the *Poxviridae* family, is the disease-causing agent. Camelpox has significant economic implications due to its high mortality rate, weight loss, reduced milk yield, and general deterioration of the condition. Clinically, two distinct types of camelpox can be recognized: the more severe generalized type, which is more common in young animals, and the less severe localized form, frequently seen in older camels (59).

During the smallpox eradication campaign, camelpox was initially considered a potential non-human reservoir of VARV (variola virus), as under particular laboratory circumstances, the two viruses exhibited no discernible differences (60). Although

CMLV only causes mild infection in humans and does not spread from person to person, there is also fear that it could be utilized as a biological weapon due to its strong genetic similarity to the variola virus (61).

The first documented case of human camelpox was reported in Somalia (34). A 40 years-old man who had not been immunized against smallpox and had come into contact with diseased animals had a rash on his arms that progressed through vesicular, pustular, and scab stages. Testing conducted at the Sera and Vaccine Institute in Mogadishu confirmed the presence of *orthopoxvirus* antibodies through a passive hemagglutination inhibition test using serum from the patient. Samples taken from sick animals in the patient's group tested positive for *orthopoxvirus* by electron microscopy, and the camelpox virus was isolated (34).

In 2009, outbreaks of dromedary camels in northwest India, reported by Bera et al. (35), provided the first strong proof of human zoonotic camelpox virus (CMLV) infection. Three human cases of CMLV zoonosis were confirmed using clinical and epidemiological attributes, serological tests, and molecular characterization of the causal agent. The camel handlers' hands and fingers were the only sites for the lesions, which developed through all stages of pock lesions until the development of scabs (35). Notably, none of the patients in the three suspected cases had received the smallpox vaccine. Serum samples from these patients revealed neutralizing antibodies against CMLV. In one of the three human cases, viral DNA specific to CMLV was detected using conventional PCR.

By describing cases involving dromedary camels and three camel herders in the Showak region of eastern Sudan between September and December 2014, Khalafalla and Abdelazim (36) offered a second piece of evidence proving the zoonotic nature of the camelpox virus. Erythema, vesicles, and pustules on the arms, hands, legs, back, and abdomen were the main skin lesions in the camel herders; they disappeared after 2 months without being spread from person to person. The diagnosis was verified by PCR, virus isolation in cell culture, and partial genome sequencing. Due to the relatively mild nature of camelpox in humans and the limited ability of the virus to spread among people, the risk to human health from this rare infection is currently considered low.

4.5.1. Current gaps and priorities for research

Events of zoonotic transmission of camelpox were documented between 1978 and 2014 in geographically remote areas of Somalia, India, and Sudan. Therefore, it is crucial to determine the factors that contributed to the emergence of the disease by isolating and sequencing CMLV strains from human patients. Studying the epidemiology of camelpox through active surveillance in regions where zoonotic transmission has occurred, such as the Showak region of eastern Sudan, is the focus of this research. Serological surveys involving dromedary camels, their shepherds, and humans who come into contact with camels in livestock markets and slaughterhouses through a one-health approach could be instrumental in tracking down and investigating zoonotic incidents.

4.6. Anthrax

Anthrax is a zoonotic bacterial disease caused by *Bacillus anthracis*, which leads to severe illness and death in humans, livestock, and wild animals. Infected animals often succumb to the disease without showing any signs of disease. Humans can contract anthrax by handling contaminated animal products, consuming undercooked meat from infected animals, and, in recent cases, the intentional release of spores.

A significant report by Musa et al. (38) provided epidemiological evidence of camel-to-human transmission of anthrax. The study investigated an anthrax outbreak in camels that resulted in 10 human infections in Darfur, western Sudan. Five affected individuals died, while the others received successful treatment. The disease was diagnosed based on human symptoms and through the Ascoli's precipitation test in camels. Control measures were implemented by mass vaccination of animals in the affected area.

In conclusion, timely diagnosis and effective control measures to mitigate the spread of this disease could be of great value. Vaccination programs are crucial in preventing anthrax outbreaks in animals and minimizing the risk of transmission to humans. Public awareness about handling and cooking animal products is essential for reducing the likelihood of human infections.

4.6.1. Current gaps and priorities for research

As demonstrated in the mentioned outbreak, the spread of anthrax from infected camels to humans highlights the importance of disease surveillance and risk assessment to establish a baseline for control measurement.

5. Additional zoonotic diseases that camels could transmit

5.1. Toxoplasmosis

Toxoplasmosis is a parasitic disease caused by *T. gondii* and is prevalent in various animals, including camels. Tonouhewa et al. (62) highlighted the widespread presence of *T. gondii* in camels, raising concerns about food safety in African countries. While the presence of *T. gondii* tachyzoites in camel's milk was documented, a direct epidemiological link to confirmed human cases was not established.

Humans can become infected with *T. gondii* through various means, such as consuming undercooked or raw meat, ingesting oocysts shed by cats through contaminated soil, food, or water, or even through transmission from mother to fetus during pregnancy. Previous studies have suggested that milk, including camel's milk, could be a source of infection. In one particular study conducted in the Butana area of eastern Sudan, researchers investigated the role of camel's milk in human toxoplasmosis. The study by Medani and Mohamed (63) presented at the 17th International Congress on Infectious Diseases examined the presence of *T. gondii* tachyzoites (a stage in the parasite's life cycle) in camel's milk. Ten milk samples from infected camels were utilized to perform an IgM anti-*T. gondii* ELISA to confirm the infection. These milk samples were then inoculated into naive kittens and mice. The results demonstrated that all the inoculated animals shed Toxoplasma oocysts, and ELISA testing confirmed the infection. However, it is essential to note that while this study suggests a possible link between camel's milk and human toxoplasmosis, it does not provide conclusive evidence. Additionally, the high seroreactivity of Toxoplasma observed among camel herders in the Butana area of eastern Sudan, as reported by Khalil et al. (64),

raises concerns about the public health implications for Sudanese nomads who consume raw camel milk.

5.2. Rift Valley fever

Rift Valley fever (RVF) is an acute disease transmitted by arthropods and caused by the RVF virus (RVFV), which belongs to the *Bunyaviridae* family and is primarily transmitted by mosquitoes. The disease was initially observed in Kenya in 1930 and has since experienced periodic outbreaks in small ruminants and cattle. These outbreaks have also led to the spread of the disease to humans in sub-Saharan Africa and the Arabian Peninsula (65). During an RVF outbreak in northeastern Kenya in 1962, camels were identified as susceptible to the virus for the first time, expanding the list of affected animal species. In September 2010, a significant RVF outbreak occurred in northern Mauritania, resulting in mass abortions among small ruminants and dromedary camels and at least 63 human clinical cases, including 13 fatalities. Among camels, the serological prevalence of the virus ranged from 27.5% to 38.5%. Notably, this outbreak marked the first clinical signs beyond abortions in camels, with animals exhibiting symptoms such as hemorrhagic septicemia and severe respiratory distress (66). During the 2022 outbreak in Mauritania, there were 47 confirmed cases of RVF, predominantly among animal breeders, with 23 fatalities reported (67). The presence of the RVF virus in animals, including small ruminants, camels, and cattle, was confirmed, with 25.8% of camel samples testing positive through RT-PCR, compared to 5.2% in cattle and 25.9% in small ruminants. This result highlighted the role played by camels in the spread and transmission of RVF, which is roughly equal to the role of small ruminants.

5.3. Tuberculosis

In a study by Gumi et al. (68), samples were collected from pastoralists in Ethiopia's Oromia and Somali Regional States with suspected tuberculosis (TB) and tuberculous lesions collected from cattle, camels, and goats at abattoirs. Culturing of humans yielded several *Mycobacterium tuberculosis* isolates, and molecular typing confirmed these isolates as *Mycobacterium bovis* and non-tuberculous mycobacteria (NTMs). Similarly, several isolates were obtained from tuberculous lesions of livestock, of which one *M. tuberculosis* and one NTM from camels. The authors concluded that the isolation of *M. tuberculosis* from livestock and *M. bovis* from humans indicates transmission between livestock and people in South-East Ethiopia's pastoral districts.

5.4. Crimean-Congo hemorrhagic fever

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne disease caused by the CCHF virus (CCHFV) that causes moderate to severe hemorrhagic disease in humans with high case fatality ratios of up to 40%. The virus spreads more quickly when infected ticks move to new, uninfected areas since the distribution of CCHFV correlates with that of its primary vector, ticks of the genus *Hyalomma*. Recently, a new lineage of CCHFV with potential genome reassortment of the M segment was found in dromedary camels and camel ticks (*Hyalomma dromedarii*) (69, 70).

5.5. Q fever (query fever, Coxiellosis)

Q fever, a significant zoonotic disease, is caused by the bacterium *Coxiella burnetii*. Infection can occur when individuals inhale aerosolized organisms or through other routes. Human infections can range from asymptomatic to causing acute, nonspecific febrile illness, often accompanied by hepatitis and atypical pneumonia (71). While there has been extensive research on the zoonotic impact of *C. burnetii* infection in small ruminant and cattle populations, the potential role of camels in transmitting Q fever to humans has received limited attention until recently (72). Given the favorable conditions for the pathogen, the Middle East region faces a significant public health threat from Q fever. Camels have an overall seroprevalence of 25% for *C. burnetii* (73). However, a study by Hussein et al. (74) in Saudi Arabia using ELISA and indirect immunofluorescence (IFA) tests found antibodies to *C. burnetii* in 51.64% of camel serum, indicating a substantially higher proportion. The authors also conducted PCR analysis on clinical samples from seropositive camels, detecting positive DNA amplification. The highest shedding of *C. burnetii* was found in fecal samples (27.59%), followed by urine (23.81%), blood (15.85%), and milk (6.5%). Based on these findings, the authors concluded that camels are a significant reservoir for *C. burnetii* and can be a primary source of Q fever transmission to humans in Saudi Arabia. In Africa, higher seropositivity rates for Q fever in camels have been reported, with 38.6%, 73.6%, and 75.5% in Kenya, Tunisia, and Algeria, respectively (75–77).

5.6. Trypanosomiasis

Trypanosoma evansi is a blood parasite found in South America, North Africa, the Middle East, and South and Southeast Asia that causes acute disease in camels and horses (surra) and chronic disease in cattle and buffalo (78). Although *T. evansi* cases in humans have been documented in India and Egypt (79), there is no epidemiological or molecular evidence that these human cases are related to the camels, in whom this parasite is known to cause a devastating and economically significant disease.

5.7. Camel contagious ecthyma

Contagious ecthyma (CE), also known as orf, represents an acute, highly infectious disease caused by different virus species of the genus *Parapoxvirus* (PPV) and family *Poxviridae*. Parapoxviruses (PPVs) commonly cause infectious skin diseases primarily affecting ruminants and other animal species, including the dromedary camel. These viruses cause proliferative exanthematous dermatitis, typified by the formation of pustules and scabs predominantly localized on the oral mucosa of afflicted animals.

It is noteworthy that the disease exhibits a more severe clinical course in camels compared to its manifestations in sheep or goats,

often culminating in a heightened case fatality rate (80). Most PPVs are thought to be zoonotic (81). Sheep-to-humans and goats-to-humans transmissions of ORFV have been documented in the literature (82, 83). Conversely, the transmission of the infection from camels to humans has not been previously reported, a position supported by empirical field investigations suggesting that CE in camels is not of zoonotic concern (80). Nevertheless, our literature examination unveiled an article detailing an intriguing case, suggesting potential camel-to-human transmission of CE (84). According to the authors, a 42 years-old male who had direct contact with a sick camel exhibited clinical manifestations consistent with orf characterized by multiple erythematous, dome-shaped to round painless nodules on the right forearm, further complicated by the occurrence of lymphadenopathy. The diagnostic determination of orf was rendered primarily based on clinical suspicion. It is noteworthy that the patient in question routinely engaged in the care of his camels and occasionally milked them. Significantly, one of his camels bore signs of CE in the form of a rash surrounding its oral cavity and both lips.

Based on the available data, classifying this case as a zoonotic camel-to-human transmission is challenging because there is no test confirmation of the suspected CE lesion on the patient and his camel. However, the publication highlighted the importance of CE and pointed out that the transmission of CE infection from camels to humans needs to be studied. Such events may go unreported because the infection is self-limiting and because those affected are frequently aware of their condition and do not seek treatment.

6. Comments on the additional zoonotic diseases that camels could transmit

In conclusion, camels have been associated with several other zoonotic diseases, including toxoplasmosis, Rift Valley fever, TB, Crimean-Congo hemorrhagic fever, Q fever, trypanosomiasis, and camel contagious ecthyma despite having no evidence of a laboratory-confirmed transmission event. These findings emphasize the need for comprehensive surveillance, preventive measures, and public health interventions based on a One Health approach to mitigate the risks of zoonotic infections linked to camels. Proper handling and processing of camel-derived products and implementing vaccination programs and vector control strategies are essential for reducing the transmission of these diseases to humans.

7. Mitigating risks and ensuring public health

To address the risks posed by camel-associated zoonotic diseases, it is necessary to increase understanding and awareness among medical professionals, veterinary authorities, and the general public. Camel herds should be under surveillance for zoonotic infections, and preventative measures like hygienic practices and appropriate food safety rules should be implemented. Collaboration between the human and animal health sectors is crucial to prevent and control zoonotic diseases.

Author contributions

The author is solely responsible for the conceptualization, design, data collection, analysis, and interpretation of the study, as well as the writing and formatting of the manuscript.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Prevalence of *Eimeria* spp. infections and major histocompatibility complex class II *DRA* diversity in Mongolian Bactrian camels (*Camelus bactrianus*)

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Introduction: The two-humped Bactrian camel (*Camelus bactrianus*) is a large, even-toed ungulate native to the steppes of Central Asia. Domestic Bactrian camels are economically important in Mongolia and other Central Asian countries. These animals are used for transport, milk and meat production, and camel racing which is a great culture of nomads. Eimeriosis, also known as coccidiosis, is considered as an economically important parasitic diseases in Bactrian camels. There is still considerable lack of data concerning the spectrum of monoxenous *Eimeria* species, their epizootiology as well as their precise life cycles in Bactrian camels. This study was performed to determine the prevalence of *Eimeria* species in camelids from southern part of Mongolia.

Methods: A total of 536 fresh camel fecal samples ($n = 536$) collected from herds located in five different Aimags (provinces) of Mongolia were examined. *Eimeria* spp. oocysts were isolated using the sugar flotation technique, and after sporulation, oocysts were identified by morphometric evaluation.

Results: We identified the most common *Eimeria* species infecting Mongolian Bactrian camels: *Eimeria cameli* (22.3%), *Eimeria rajasthani* (37.3%) and *Eimeria dromedarii* (27.7%). Interestingly, mixed infections were detected in 24.8% ($n = 133$) of the samples, while 39.0% ($n = 209$) were negative for coccidian stages. To investigate the immunogenetic response of the Mongolian Bactrian camels to *Eimeria* spp. infection, we screened the genetic diversity in a functional important immune response gene of the major histocompatibility complex (MHC). We detected two polymorphic sites in the MHC class II *DRA* exon 2, which translated into one non-synonymous and one synonymous amino acid (aa) change.

Discussion: The resulting aa alleles were not significantly associated with any of the three detected *Eimeria* species infections, nor could we show heterozygote advantage in non-infected Mongolian Bactrian camels. Further investigations on molecular epidemiology, *in vitro* culture, pathogenicity and host–parasite interactions will be necessary to better understand the impact of eimeriosis in Bactrian camels.

KEYWORDS

Camelus bactrianus*, coccidiosis, *Eimeria cameli*, *Eimeria rajasthani*, *Eimeria dromedarii

Introduction

Extant two-humped camel species are represented by the domestic (*Camelus bactrianus*) and wild (*Camelus ferus*) species. At present, wild camels are the only wild survivors of the Camelini tribe and inhabit northwestern China and southwestern Mongolia, especially within the Outer Altai Gobi Desert (1, 2). Domestic Bactrian camels are mainly distributed in the arid desert of Asian countries, such as Mongolia, China, Russia, Kazakhstan and Iran (3). These large animals are economically important in Mongolia where they are used for transport, entertainment (camel race, camel polo), and production of derived products such as fermented milk, meat, wool and skin (4), justifying the great socioeconomic importance of camels in the country.

Eimeriosis, also known as coccidiosis, is considered an important parasitic enteric disease of camels (5), but the occurrence of monoxenous (that lives within a single host during its whole life cycle) (6) *Eimeria* species and prevalence of eimeriosis is unknown in Bactrian camels in Mongolia. Among the five species known to infect Bactrian camels, i. e. *Eimeria cameli*, *E. rajasthani*, *E. dromedarii*, *E. bactriani* and *E. pellerdyi*. Nonetheless, *E. cameli*, *E. rajasthani* and *E. dromedarii* are considered as the most pathogenic species forming first generation macromeronts as reported for other highly pathogenic *Eimeria* species of domestic ruminants and New World camelids (7–10). Several studies showed the prevalence of different *Eimeria* species in camels. As such, Chineme (11) reported a case of dromedary (*Camelus dromedarius*) coccidiosis caused by *E. cameli* in Nigeria (11). In other studies, Kawasmeh and Elbihari (12), Yagoub (13), and Kasim et al. (14) found one or more species (*E. rajasthani, E. dromedarii* and *E. cameli*) with an overall prevalence of 14% in Saudi camels (*C. dromedarius*), 17.4% in Sudanese camels (*C. dromedarius*) and 41.6% in Saudi Arabian camels (*C. dromedarius*), respectively (12–14).

The report by Tafti et al. (15) indicated that the most important and frequent pathologic lesion in the digestive tract of camels is resulting from *Eimeria* spp. infections (63% of 100 slaughtered camels) (15). These pathological findings were in close agreement with reports from Hussein et al. (16), Kasim et al. (14) and Borji et al. (17) (14, 16, 17). Several cases of eimeriosis causing enteritis and mortality rates of up to 10% in young camels have been reported in few cases (11, 18, 19). *Eimeria cameli, E. rajasthani, E. dromedarii* are pathogenic to young camel calves causing enteritis (16). Infected young animals showed wasting, debility and diarrhea without mucus or blood. Older animals shedding oocysts in their faeces did not show any serious symptoms of eimeriosis (20). Considering the fact that all *Eimeria* infections are highly host and host cell-specific and that stage-specific innate as well as adaptive immune reactions are a common feature (21, 22), it appears essential that basic research is performed on different developmental stages in the respective hosts (8, 23).

Pathogen-mediated selection has been described as a driver for genetic diversity in host immune response genes, especially in the major histocompatibility complex (MHC). The MHC class I and class II genes are responsible for encoding molecules on the cell surface that recognize and present antigens (24). Therefore, these molecules are under strong selective pressure and have an important role for the adaptive immune response and for host-pathogen co-evolution (25). Studies in Inner Mongolian Brandt's voles showed that MHC class II diversity is maintained by rare allele advantages and fluctuating selection, and that the association between intestinal parasite load and specific MHC class II *DRB* alleles varied between geographical regions (26).

In the three extant Camelini species (*C. bactrianus*, *C. ferus*, *C. dromedarius*), the MHC is located on chromosome 20, with the class II region located closer to the centromere and the class I more distant (27). In camels, unexpectedly low diversity has been described in the MHC class II loci (27) as well as in all functional different groups (adaptive and innate) of immune response genes (28). So far, no study about the immunogenetic response in camels to intestinal parasite infection has been conducted.

To fill this knowledge gap, the aim of this study was to determine the prevalence of *Eimeria* species in domestic Bactrian camels in southern Mongolia as well as their adaptive immunogenetic response to *Eimeria* spp. infections. Due to its functional importance, specifically in connection with parasite infection, we focused on the exon 2 coding sequence of the MHCII *DRA* locus and investigated pathogen-mediated immunogenetic diversity in Mongolian Bactrian camels infected or non-infected with *Eimeria* spp.

Materials and methods

Sample collection and study field area

Fecal samples were collected from 536 Mongolian Bactrian camels in the Umnugobi-, Bayankhongor-, Uvurkhangai-, Dundgobi– and Khovd Aimags, chosen at random (Figure 1). Collected samples were put separately into closed plastic containers and identified with numbers. In addition, 100 EDTA peripheral blood samples were collected in parallel to the fecal sample collection. All experimental protocols were approved by the Animal Care and Use Committee, Institute of Veterinary Medicine, Mongolian University of Life Sciences (MULS) (Agreement Number № MEBUS-16/01/05). Samples from local provinces were taken from live animals with official permission and under the supervision of the Provincial Veterinary Organization in accordance with the regulation of the Animal ethic committee, MULS.

The examined Bactrian camels followed traditional husbandry practices, with animals grazing during daytime. Camels were mainly crossbreeding and indigenous. For representative reasons, geographical locations of camels sampled are shown in Figure 1.

Detection and identification of *Eimeria* oocysts

All fecal samples were examined by sugar flotation technique for isolation of camelid *Eimeria* spp. oocysts. Briefly, 3 g of fecal material were weighted and placed into a beaker and 15 mL of saturated sucrose solution (Sheather's solution, specific gravity = 1.28) were added and homogenized. Thereafter, the fecal suspension was transferred into a 15 mL centrifuge tube and centrifuged at 2,000 rpm for 10 min at room temperature (RT) (29–37).

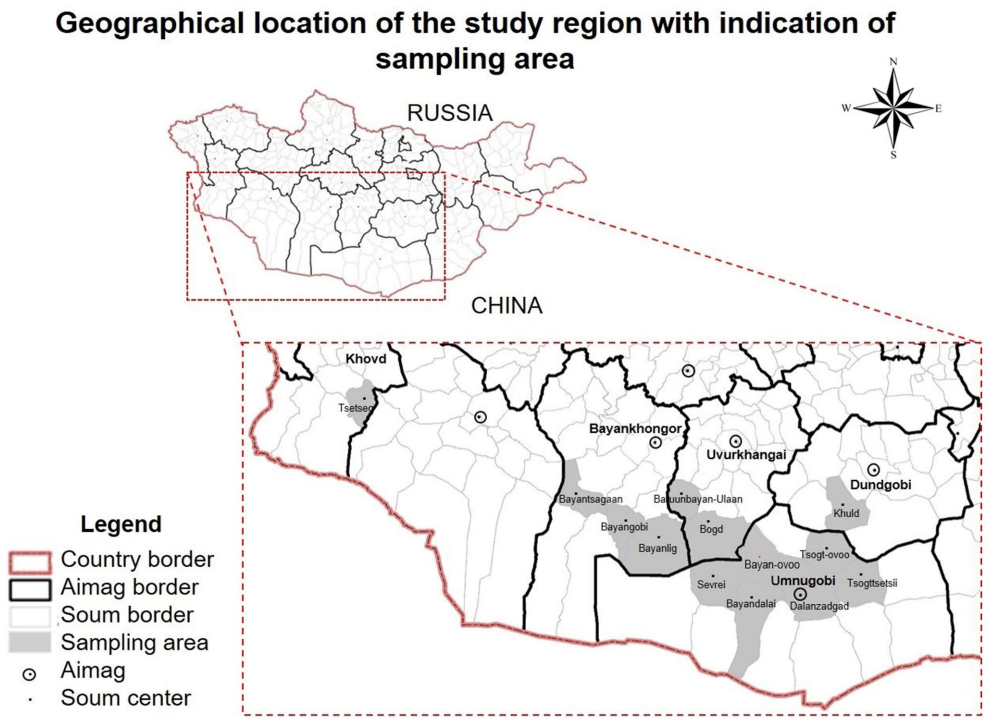

FIGURE 1
Geographical location of the study region with indication of Bactrian camel (*Camelus bactrianus*) sampling areas. Khovd, Bayankhongor, Uvurkhangai, Dundgobi, Umnugobi Aimags of Mongolia. This map has been provided by the ArcGIS 10.2 program.

Samples were investigated by means of light microscopy and all oocysts within the microscope slides were considered in this study.

For species identification, oocysts from each individual sample were allowed to sporulate in 2.5% potassium dichromate under constant oxygenation (38). *Eimeria* species identification was based on the morphological and morphometric features of sporulated oocysts such as the size, shape, colour and texture of oocyst wall, presence or absence of micropyle, polar cap, among others, with the aid of taxonomic keys according to Levine and Ivens (1970), (6, 29, 39).

Analysis of the major histocompatibility complex (MHC) class II *DRA* exon 2

DNA was extracted using the QIAmp® blood mini kit (Qiagen, Vienna, Austria) from 100 EDTA peripheral blood samples following the manufacture's instruction. The 246 bp long MHCII *DRA* exon 2 was amplified with the camel specific DNA primer pairs (DRA-ex2-F-TGAGAATTTTGGGTTTGCTTATGGCA/ DRA-ex2-R-CCTCTGAGCAACACG AACGTC CTTCA) with an annealing temperature of 57°C (27). The PCR reactions were performed in a reaction volume of 15 µL including 0.2 mM dNTPs, 25 mM $MgCl_2$, 0.5 µM of forward and reverse primer, 1x Amplitaq Gold buffer, and 0.5 U of Amplitaq Gold Hotstart polymerase (ThermoFisher Scientific, Vienna, Austria). Please note that we tried to sequence also MHCII *DRB* exon 2 following Plasil et al. (27), however, only few samples yielded PCR products, which might be due to the longer amplicon of 852 bp (27). Successfully amplified PCR products were purified with FastAP™ and Exonuclease I (ThermoFischer Scientific) following the manufacture's guide for PCR product clean-up prior to sequencing. The purified PCR products were Sanger sequenced in both directions with the BigDye™ Terminator v3.1 Cycle Sequencing Kit (ThermoFischer Scientific) on an ABI sequencer at the Research Institute of Wildlife Ecology, Vetmeduni Vienna, Austria. Sequences were visualised, aligned and translated into amino acids (aa) using CodonCode Aligner 11.0.1 (CodonCode Cooperation, Centerville, USA). We applied DNAsp 5.10.1 (40) to phase ambiguous (heterozygous) sequences and to determine haplotype (Hd) and nucleotide diversity (Pi).

To test for potential associations between *Eimeria* infection and the MHCII *DRA* exon2 aa alleles as well as for heterozygote advantage, we applied the generalized linear model with a logit link function in IBM® SPSS® Statistics version 29.0.1. (IBM Corp., Armonk, NY, USA). We tested six models with each of the three detected *Eimeria* species as binary dependent variable and the aa alleles, heterozygosity, gender and the respective other *Eimeria* spp. (Table 1) as binary, location (soum) as categorical, and age as continuous predictor variables, respectively. We applied strict Bonferroni correction for multiple model (k = 6) testing for a nominal alpha error of 0.05. In case of a significant effects of co-infection with two *Eimeria* species ($p < 0.0083$, Bonferroni corrected) we used Crosstabs statistics in SPSS to calculate the association coefficient Phi, which is a chi-square-based measure of association between nominal data (41).

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TABLE 1 Sample information for genotyped (MHC II *DRA* exon 2) individuals.

ID	Genotype MHCII *DRA* exon 2 pos.58/143	aa* allele	*E. cameli*	*E. rajasthani*	*E. dromedarii*	soum	gender	age
T-1	TT/GG	0	0	0	0	Bayan-Ovoo	female	5
T-2	TA/GT	2	0	0	0	Bayan-Ovoo	female	2
T-3	TT/TT	0	0	0	0	Bayan-Ovoo	female	6
T-5	TT/GG	0	0	0	0	Bayan-Ovoo	female	6
T-6	TT/GG	0	0	0	0	Bayan-Ovoo	female	10
T-7	AA/TT	1	0	0	0	Bayan-Ovoo	female	8
T-8	AA/TT	1	0	0	0	Bayan-Ovoo	female	9
T-9	TT/GG	0	0	0	0	Bayan-Ovoo	female	10
T-10	TA/GG	2	0	0	0	Bayan-Ovoo	female	1
T-11	TA/TT	2	0	0	0	Bayan-Ovoo	female	6
T-12	TT/GG	0	0	0	1	Bayan-Ovoo	female	6
T-13	TT/GG	0	0	1	1	Bayan-Ovoo	female	9
T-14	TT/GG	0	1	0	1	Bayan-Ovoo	female	7
T-15	TT/GG	0	1	1	0	Bayan-Ovoo	female	7
T-23	TA/TT	2	1	1	0	Bayan-Ovoo	female	1
T-24	TA/GT	2	0	0	0	Bayan-Ovoo	male	1
T-25	TA/GT	2	0	0	0	Bayan-Ovoo	female	1
T-26	TA/GT	2	0	0	0	Bayan-Ovoo	female	1
T-27	TA/GT	0	0	0	0	Bayan-Ovoo	male	1
T-28	TA/GT	2	0	0	0	Bayan-Ovoo	male	1
T-29	TA/GT	2	0	0	0	Bayan-Ovoo	female	1
T-30	TA/GT	2	0	0	0	Bayan-Ovoo	female	1
T-31	AA/TT	1	0	0	0	Tsogttsutsii	female	10
T-32	TA/GT	2	0	1	1	Tsogttsutsii	male	1
T-33	TA/GT	2	0	1	1	Tsogttsutsii	female	8
T-34	TA/GT	2	1	0	1	Tsogttsutsii	female	8
T-35	AA/TT	1	0	0	1	Tsogttsutsii	female	8
T-36	AA/TT	1	0	0	1	Tsogttsutsii	female	6
T-37	TT/GG	0	0	0	1	Tsogttsutsii	female	6
T-38	TA/GT	2	0	0	1	Tsogttsutsii	female	7
T-46	AA/TT	1	0	0	0	Tsogttsutsii	male	1
T-54	TT/GG	0	0	0	0	Tsogttsutsii	female	14
T-55	TA/GT	2	0	0	0	Tsogttsutsii	female	15
T-56	TA/TT	2	0	0	0	Tsogttsutsii	female	14
T-57	TA/GT	2	0	0	0	Tsogttsutsii	female	6
T-58	TT/GG	0	0	0	0	Tsogttsutsii	female	14
T-59	TT/TT	0	0	0	0	Tsogttsutsii	female	20
T-60	TA/TT	2	0	0	0	Tsogttsutsii	female	14
T-61	TA/GT	2	0	0	0	Tsogttsutsii	female	6
T-62	AA/TT	1	0	0	0	Tsogttsutsii	female	14
T-63	TA/GT	2	0	0	0	Tsogttsutsii	female	6
T-64	TA/GT	2	0	0	0	Dalanzadgad	male	1

(Continued)

TABLE 1 (Continued)

ID	Genotype MHCII *DRA* exon 2 pos.58/143	aa* allele	*E. cameli*	*E. rajasthani*	*E. dromedarii*	soum	gender	age
T-65	TT/GT	0	0	0	0	Dalanzadgad	female	1
T-66	TA/GT	2	0	0	0	Dalanzadgad	female	1
T-67	TA/GT	2	0	0	0	Dalanzadgad	female	1
T-68	TT/GG	0	1	1	0	Dalanzadgad	male	1
T-69	TA/TT	2	0	0	0	Dalanzadgad	male	1
T-70	TT/GT	0	0	0	0	Dalanzadgad	female	1
T-71	TA/GT	2	0	0	1	Dalanzadgad	male	1
T-72	TT/GT	0	0	0	1	Dalanzadgad	female	1
T-73	TA/GT	2	1	1	0	Dalanzadgad	female	1
T-74	TA/TT	2	0	0	1	Dalanzadgad	female	12
T-76	AA/TT	1	0	0	1	Dalanzadgad	female	5
T-77	AA/TT	1	0	0	1	Dalanzadgad	female	10
T-78	AA/TT	1	0	0	0	Dalanzadgad	female	12
T-79	TA/TT	2	0	0	0	Dalanzadgad	female	12
T-80	TA/GT	2	0	0	0	Dalanzadgad	female	5
T-81	TA/TT	2	0	0	0	Dalanzadgad	female	5
T-82	TA/TT	2	0	0	0	Dalanzadgad	female	5
T-84	TA/GT	2	0	0	0	Dalanzadgad	female	14
T-85	TA/GT	2	0	0	0	Dalanzadgad	female	6
T-86	TA/TT	1	0	0	0	Dalanzadgad	female	5
T-87	TA/GT	2	0	0	0	Dalanzadgad	female	10
T-88	TA/TT	2	0	0	0	Dalanzadgad	female	21
T-89	TT/TT	0	0	0	0	Dalanzadgad	female	7
T-90	TA/GT	2	0	0	0	Dalanzadgad	female	6
T177	TA/TT	2	0	1	0	Bayangobi	female	3
T178	TT/GT	2	0	0	0	Bayangobi	female	11
T179	TT/GT	2	0	0	0	Bayangobi	female	1
T180	TT/GT	2	0	0	0	Bayangobi	female	9
Eimeria spp. prevalence (%)			6 (8.6)	8 (11.4)	15 (21.4)			

*0: homozygote H1 (FF); 1: homozygote H2 (YY); 2: heterozygote H1/H2 (FY).

Results

Eimeria spp. infection in Mongolian Bactrian camels

In total, 327 fecal samples ($n = 327$) had *Eimeria* oocysts with an overall prevalence of 61% in Bactrian camels from Bayankhongor-, Uvurkhangai-, Umnugobi-, Dundgobi– and Khovd Aimags. *Eimeria* parasites were found in all five investigated Aimags (Figure 1). Three different camelid-specific *Eimeria* species were identified, being *E. rajasthani* 200 (37.3%) and *E. dromedarii* 149 (27.7%) the most prevalent ones, followed by *E. cameli* 120 (22.3%). In 209 samples (39%) no *Eimeria* oocysts were observed (Table 2; 3). Mixed *Eimeria* spp. infections with two or three *Eimeria* species, were detected in 133 samples (24.8%) (Table 3). Out of 327 positive samples, 194 (36.1%) samples presented single infection, 125 (23.3%) samples had two species, and only 9 (1.6%) samples had mixed infections with all three species (see Figure 2).

MHC II *DRA* diversity in Mongolian Bactrian camels with *Eimeria* spp. infections

We successfully amplified and sequenced the 246 bp long MHC class II *DRA* exon 2 in 70 (out of 100) samples (Table 1). We screened the *DRA* exon 2 for genetic diversity and detected two polymorphic nucleotides (nt) at the positions nt = 58 and nt =143 in the 246 bp long fragment. Phasing the 70 individual

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TABLE 2 Occurrence of *Eimeria* spp. in Mongolian Bactrian camels.

Name of Aimag	Soum	Sample number	*E. cameli*		*E. rajasthani*		*E. dromedarii*		Mixed infection	
			Positive	%	Positive	%	Positive	%	Positive	%
Bayankhongor	Bayantsagaan	82	23	28.05	29	35.3	15	18.29	16	19.51
	Bayanlig	60	10	16.67	45	75	30	50	31	51.6
	Bayangobi	14	4	28.57	7	50	9	64.29	8	57.14
Total		156	37	23.72	81	51.92	54	34.62	55	35.9
Uvurkhangai	BaruuBayan-Ulaan	85	20	23.53	33	38.82	26	30.59	21	24.71
	Bogd	17	10	58.82	6	35.29	1	5.88	3	17.65
Total		102	30	29.41	39	38.24	27	26.47	24	23.53
Umnugobi	Sevrei	65	23	35.38	26	40	23	35.38	18	27.69
	Dalanzadgad	37	6	16.21	6	16.21	12	32.43	6	16.21
	Tsogttsutsii	49	3	6.12	8	16.32	17	34.69	9	18.36
	Tsogt-Ovoo	21	1	4.76	1	4.76	2	9.52	1	4.76
	Bayandalai	32	5	15.63	17	53.13	1	3.13	4	12.5
	Bayan-Ovoo	30	4	13.33	7	23.33	9	30	8	26.66
Total		234	42	17.94	65	27.77	64	27.35	46	19.65
Dundgobi	Khuld	19	10	52.63	4	21.05	1	5.26	4	21.05
Khovd	Tsetseg	25	1	4	11	44	3	12	4	16
Grand total		536	120	22.3	200	37.3	149	27.7	133	24.8

TABLE 3 Mixed *Eimeria* spp. infection in Mongolian Bactrian camels.

Name of Aimag	Soum	total	Mixed infection									
			Number of mixed infections		non infected		single species		two species		three species	
			Pos.	%	Neg.	%	Pos.	%	Pos.	%	Pos.	%
Bayankhongor	Bayantsagaan	82	16	19.5	33	40.2	33	40.2	14	17	2	2.4
	Bayanlig	60	31	51.6	7	11.6	22	36.6	29	48.3	2	3.3
	Bayangobi	14	8	57.1	2	14.3	4	28.6	8	57.1		
Total		156	55	35.2	42	26.9	59	37.8	51	32.6	4	2.6
Uvurkhangai	Baruunbayan-Ulaan	85	21	24.7	31	36.4	33	38.8	18	21.1	3	3.52
	Bogd	17	3	17.6	3	17.6	11	64.7	3	17.6		
Total		102	24	23.5	34	33.3	44	43.1	21	20.5	3	2.9
Umnugobi	Sevrei	65	18	27.6	11	16.9	36	55.3	18	27.6		
	Dalanzadgad	37	6	16.21	21	56.8	10	27.02	5	13.51	2	5.4
	Tsogttsutsii	49	9	18.3	30	61.2	10	20.4	9	18.3		
	Tsogt-Ovoo	21	1	4.76	18	85.7	2	9.5	1	4.7		
	Bayandalai	32	4	12.5	13	40.6	15	46.8	4	12.5		
	Bayan-Ovoo	30	8	26.6	18	60	4	13.3	8	26.6		
Total		234	46	19.7	111	47.4	77	32.9	45	19.2	2	0.9
Dundgobi	Khuld	19	4	21	8	42.1	7	36.8	4	21		
Khovd	Tsetseg	25	4	16	14	56	7	28	4	16		
Grand total		536	133	24.8	209	39.0	194	36.2	125	23.3	9	1.7

sequences resulted in three haplotypes (h = 3) with a haplotype (gene) diversity Hd = 0.620 (± 0.018), nucleotide diversity Pi = 0.004 (± 0.00006) and an average number of nucleotide differences k = 0.983. At position nt = 58, the change from T to A (T58A) led to a non-synonymous amino acid (aa) change from phenylalanine (F) to tyrosine (Y), both hydrophobic, while the nucleotide change G143T was synonymous (no aa change). This resulted in two different aa alleles (haplotypes; H1 and H2) identified in Mongolian Bactrian camels as shown in Figure 3. While 19 camels were homozygous for H1 and 11 individuals for H2, respectively, the majority of 40 Bactrian camels was heterozygous and harboured both aa alleles of the MHCII *DRA* exon 2. The complete sample, genotype and aa allele information is presented in Table 3. The sequence alignment of MHCII *DRA* exon 2 for all Bactrian camel samples is provided in Supplementary file S1.

We investigated a potential statistical effect between the MHC *DRA* exon 2 aa alleles H1, H2 or H1/H2 on the three different *Eimeria* spp. infections in the Bactrian camels, using a generalised linear model approach. However, we could not identify any significant ($p < 0.0083$ after Bonferroni correction) association between the MHCII *DRA* aa alleles and any of the *Eimeria* spp. infections (Table 4). Similarly, we did not detect a significant effect of the homozygote or heterozygote genotypes on *Eimeria* spp. infections, respectively. However, we identified a significant ($p = 0.003$) positive effect with a moderate association coefficient Phi = 0.532 ($p < 0.001$) between two *Eimeria* species, *E. cameli* and *E. rajasthani*, independent from all other tested predictor variables and factors, i. e., location, age or gender did not show any significant effect on the prevalence of *E. cameli* and *E. rajasthani* in the respective models under strict Bonferroni correction (Table 4).

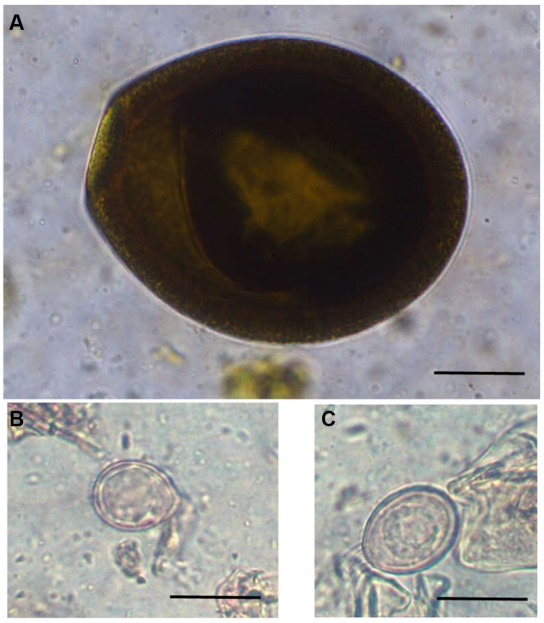

FIGURE 2
Photomicrographs of different unsporulated *Eimeria* oocysts of Bactrian camels (*Camelus bactrianus*) in Mongolia. **(A)** *Eimeria cameli*. The oocysts were truncated ovoid, dark brown to black in colour. The oocyst wall was composed of 3 layers: outer, dark brown in colour with tiny projections. The middle layer was thin smooth and yellowish in colour. The inner layer was dark brown. Length x width 96.5 × 82 µm, **(B)** *Eimeria dromedarii*. The oocyst shape ranged from subspherical to ovoid, with rough walls composed of two distinct layers: outer, pale yellow and inner, dark green. Length x width = 23 × 17 µm and, **(C)** *Eimeria rajasthani*. The oocysts were ellipsoidal in shape with smooth walls that were composed of two layers: outer, pale yellow and inner, yellowish green in colour. The size length x width 29 × 21.6 µm. Scale bar = 25 µm.

Discussion

Eimeriosis, in Camelini worldwide has been recently summarized (5). However, no reports from Mongolia were included since there were no available studies at the time. Here, we report the prevalence of *Eimeria* spp. infections in southern Mongolia. Due to the rather high prevalence of camel coccidiosis, it could be assumed that *Eimeria* infections are widely spread in the country, and it may play an important role as underestimated subclinical or clinical disease affecting the growth rate performance of mainly young Mongolian Bactrian camels as reported for other hosts. *Eimeria* spp. prevalence up to 50% was reported in Bactrian camels from Inner Mongolia in China (42). In total, three monoxenous *Eimeria* species were found: *E. dromerdarii*, *E. rajasthani* and *E. cameli*, which are considered pathogenic for camels (20). Mixed infections with two or three species were here observed, presenting a higher frequency then previously reported by Yakhchali and Athari (2010) (43). The authors described the identification of four *Eimeria* species including *E. bactriani* (52.4%), *E. cameli* (19.3%), *E. pellerdyi* (15.6%) and *E. dromedarii* (12.5%) and mixed infections (up to four *Eimeria* species) in 10.54% of investigated camels.

Regarding pathogeny of camel coccidiosis several studies confirm its importance. Iyer et al. (44) reported an outbreak of gastroenteritis in camels in Punjab, India, affecting hundreds of camels with 1–40% mortality during summer months (44). Two dead camels were examined at necropsy. Gastroenteritis was the predominant finding and affected abomasum, duodenum, and cecum; jejunum and ileum were not examined. Endogenous stages (schizonts, gamonts, and oocysts) were detected in duodenum, and cecum (15, 45, 46). Same conclusion applies to a similar case of haemonchosis and *E.*

H1: EHVIIQAEFYLNPDKSGE**F**MFDFDGDEIFHVDLEKKETVWRLEEFGRFASFEAQGALANMAVDKANLDIMMKRSNHTPNTN

H2: EHVIIQAEFYLNPDKSGE**Y**MFDFDGDEIFHVDLEKKETVWRLEEFGRFASFEAQGALANMAVDKANLDIMMKRSNHTPNTN

FIGURE 3
MHCII *DRA* exon 2 amino acid haplotypes identified in Mongolian Bactrian camels. The polymorphic amino acid change is highlighted in bold.

TABLE 4 Parameter estimates of the generalized linear model testing between MHCII *DRA* exon 2 amino acid (aa) alleles and *Eimeria* spp. infections.

			Dependent variable								
			E. cameli			*E. rajasthani*			*E. dromedarii*		
Parameter estimates			B	Std.err	Sig.	B	Std.err	Sig.	B	Std.err	Sig.
Predictor variables		Intercept	0.497	1.705	0.771	−3.681	1.938	0.058	−0.225	1.334	0.866
	Eimeria sp.	*E. cameli*	_	_	_	3.473	1.166	0.003	0.047	1.214	0.969
		E. rajasthani	3.467	1.166	0.003	_	_	_	1.301	1.115	0.243
		E. dromedarii	0.440	1.435	0.759	1.141	1.281	0.373	_	_	_
	aa allele	H0	−0.847	1.332	0.525	−0.414	1.218	0.734	−0.953	0.764	0.213
		H1	18.726	23,468	0.999	19.736	21,802	0.999	−1.233	0.849	0.147
		H2	0[a]	.	.	0[a]	.	.	0[a]	.	.
		homozygous	−0.586	1.342	0.662	0.173	1.112	0.877	1.068	0.664	0.108
		heterozyous	0[a]	.	.	0[a]	.	.	0[a]	.	.
	Soum	Bayan-Ovoo	−0.472	2.022	0.815	0.874	1.658	0.598	1.850	0.93	0.047
		Bayangobi	20.818	32,680	0.999	−1.598	1.906	0.402	22.21	37,402	1.000
		Dalanzadgad	−0.350	2.165	0.817	1.529	1.786	0.392	1.093	0.821	0.183
		Tsogttsutsii	0[a]	.	.	0[a]		.	0[a]	.	.
	Sex	female	−1.325	1.803	0.463	0.771	1.345	0.566	−0.745	1.144	0.515
		male	0[a]	.	.	0[a]		.	0[a]	.	.
		age	0.141	0.201	0.485	0.191	0.184	0.298	0.086	0.086	0.321

B: unstandardised coefficient (slope of the line between the predictor and dependent variables).[a]set to zero by SPPS because this parameter is redundant (can be explained by the other variables).Sig.: *p*-values; highlighted in bold if significant ($p < 0.0083$) after strict Bonferroni correction for multiple model (k = 6) testing with a nominal alpha error of 0.05.

cameli-associated gastroenteritis in one year old camel from India (45). Rangarao and Sharma (47) noted diarrhea-associated with the presence of *E. rajasthani* oocysts in all eight calves in India (47). An eimeriosis-like illness was diagnosed histologically in 27 of 38 camels submitted in 1996 for *postmortem* examination to the Central Veterinary Research Laboratory (CVRL), Dubai, UAE (48). Of these 27, illness was severe in 21 and mild in 6 animals, respectively. Severe hemorrhagic enteritis with eosinophilia of small intestine (mostly jejunum and ileum and rarely duodenum) was associated with numerous stages of *E. cameli* whereas large intestines were not affected.

Camel eimeriosis has mostly been associated with younger camels (20, 49). It is an important disease in pre-weaned and recently weaned camels (20). While nearly animals of all ages are exposed to infectious sporulated *Eimeria* oocysts in the environment, they may not show obvious signs of the disease. In the majority of the hosts, the parasite coexists causing minimal damage to the infected host (20). Clinical eimeriosis usually occurs if the host is subject to a heavy infection, with high number of infectious oocysts ingested, or if its resistance is lowered (20), and its immune status is not adequate to cope with a coccidian infection.

Another critical time for the infection of camels could be the time immediately preceding the period of dryness (peak from July to October), when the short winter rains cause camels to crowd from the surrounding desert to limited water holes and springs in oases (12), like in Saudi Arabia, potentially promoting the spread of the disease. Further studies with camel *Eimeria* spp., including molecular characterization and establishment of suitable *in vitro* culture systems will allow detail investigations on sporozoite-host cell interactions and early host innate immune reactions as reported for ruminant eimeriosis (8, 38).

Concerning immunogenic response, in the 246 bp exon 2 of the MHCII *DRA* locus sequenced in 70 Mongolian Bactrian camels infected or non-infected with *Eimeria* spp. we detected the same two nucleotide polymorphisms as described before in a global set of Bactrian camels (27). These synonymous and non-synonymous polymorphisms, which produce three different aa alleles are also shared between dromedaries (*C. dromedarius*) and wild camels (*C. ferus*) (27). The frequency (0.57) of the heterozygous allele in the here investigated Mongolian Bactrian camels was similar to the frequency described in global Bactrian camels (0.53), lower than in wild camels (0.63) and higher than in dromedaries (0.32) (27). MHC diversity is often maintained by pathogen-mediated balancing selection. It is generally assumed that heterozygous individuals have an advantage, e.g., higher fitness than individuals that are homozygous at the same locus (50, 51). Although we observed twice as many heterozygous individuals ($n = 40$) for the aa alleles at position nt 58 than homozygotes for either the reference ($n = 19$) or alternative ($n = 11$) allele, we could not identify a statistically significant heterozygosity effect on the *Eimeria* spp. infection, in terms of prevalence. No evidence for MHC class II *DRB* heterozygote advantage in relation to intestinal parasite infection has been described in Brandt's voles from Inner Mongolia (26). The lack of such a heterozygosity effect in our study might also be explained by the relatively low number ($n = 70$) of successfully phenotyped (*Eimeria* spp. infection) and genotyped (MHCII *DRA* exon 2) samples. In addition, the *Eimeria* infection status was evaluated in a qualitative way, i.e., presence or absence,

while a quantitative assessment (i.e., intensities of infection) would have provided more refined infection data to be included into our statistical models. Contrary to many other species, Old World camels and specifically Bactrian camels have a low number of MHC II *DRB* alleles. In fact, the *DRB* exon 2 showed only four alleles in 43 previously investigated Bactrian camels, which translated into two haplotypes at the amino acid level with one haplotype present in 74% of the samples (27). As our attempt to amplify MHCII *DRB* exon 2 unfortunately failed, we cannot exclude that we might have identified new *DRB* alleles in the investigated Mongolian Bactrian camels. However, considering our findings of the *DRA* exon 2 identifying exactly the previously described alleles (27), we probably might neither expect novel *DRB* alleles in the population. We did not find any age-associated effect on *Eimeria* spp. prevalence, even though older individuals may be expected to have higher prevalence than younger ones, simply due to the longer lifetime that accumulates their change of infection. This might suggest that adult may cope with the infection successfully and clear it, possibly also in connection with their overall immunogenetic status.

Interestingly, we detected a moderate positive association between the two *Eimeria* species *E. cameli* and *E. rajasthani*. Co-evolution between two parasites (*E. cameli* and *E. rajasthani*) has been observed, and these two species were dominant in Mongolian camels. While at a low dose inoculate a linear reproduction is observed, at higher doses the reproduction of the parasite becomes impaired (the so-called 'crowding effect'), mainly due to the damage of cells or lower availability of nutrients (52). Resistance of the host to a pathogen is a very important factor in the evolutionary arms race between host and parasite. Although the host evolves at a much slower rate than the parasite is capable of, the host has developed ways to reduce susceptibility to infection. We argue that studying questions of host–parasite interaction in camelids can be well approached with an *Eimeria* parasite infection system in Bactrian camels.

In conclusion, this study revealed that *E. cameli, E. rajasthani,* and *E. dromedarii* infections frequently occur in Mongolian Bactrian camel. Given that clinical and subclinical *Eimeria* spp. infections are well known to dampen camel production, regular monitoring including diagnosis of species, quantitative description of the parasite infection load, and MHC and other immunogenetic loci could help to prevent future *Eimeria*-induced economic losses in Mongolian camel rearing.

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.

Ethics statement

The animal studies were approved by Animal Care and Use Committee, Institute of Veterinary Medicine, Mongolian University of Life Sciences (Agreement Number № MEBUS-16/01/05). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

IK: Conceptualization, Formal analysis, Funding acquisition, Writing – original draft, Data curation, Investigation. UN: Data curation, Investigation, Visualization, Writing – review & editing. BC: Data curation, Investigation, Writing – review & editing. KN: Data curation, Formal analysis, Investigation, Writing – review & editing. AT: Conceptualization, Supervision, Validation, Writing – review & editing. CH: Conceptualization, Supervision, Validation, Writing – review & editing. FS: Formal analysis, Methodology, Writing – review & editing. FK: Methodology, Software, Validation, Writing – review & editing. PB: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft. GB: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1296335/full#supplementary-material

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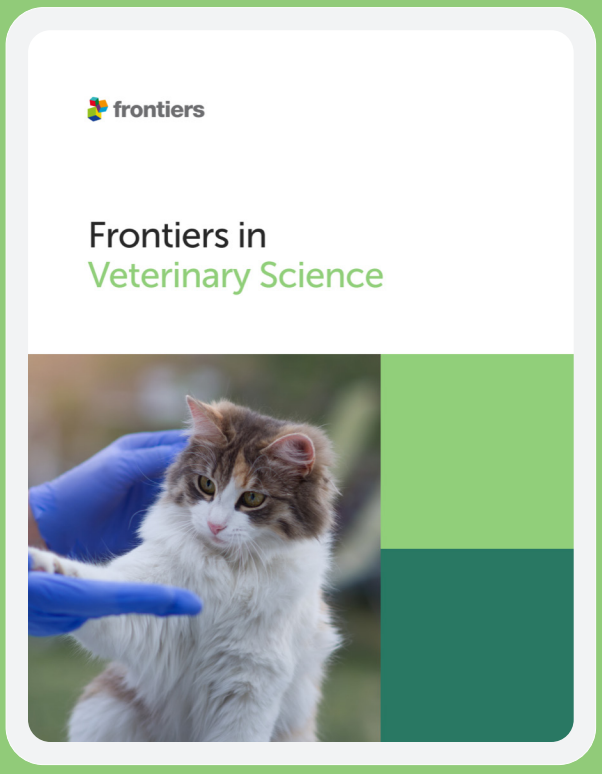

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