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Zoonoses - a one health approach

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Zoonoses - a one health approach

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Editorial: Zoonoses - a one health approach

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Editorial on the Research Topic
Zoonoses - a one health approach

Zoonoses are defined as "those diseases and infections [*the agents of*] which are naturally transmitted between [*other*] vertebrate animals and man" (1). Zoonotic diseases are responsible for considerable negative health impacts and economic consequences (2). Many recently discovered emerging pathogens, such as SARS-CoV-2, Ebola, and Nipah virus, are zoonotic in nature (3), and the cross-species pathogen spillover from non-human animal hosts to humans could be associated with the majority of human infectious diseases and pandemics (4–6). An increase in human–animal interactions, contacts, and the human–animal interface may increase the risk of zoonotic pathogens in manifold human populations (7). Furthermore, societal characteristics, ecological disturbances, environmental stress, and climate change have strong association with zoonoses (8–11). In contrast to the diseases caused by single-host pathogens, prevention, control, and eradication of the diseases caused by zoonotic pathogens (which involve two or more hosts) are often difficult due to the complex pathogen transmission matrix and the real probability of the presence of multiple reservoir hosts. Interventions at the critical control points (CCPs) of zoonotic diseases are essential and need multi-sectoral engagement. Formal or informal slaughterhouses, wet markets, and carcass disposal sites are important CCPs that need attention at the animal–environment interface. A lack of coordination among environmental, animal, and public health sectors, nationally and internationally, has the potential to seriously undermine zoonotic disease control programs. In addition, antimicrobial resistance (AMR) has complicated the prevention and control of zoonoses due to the emerging general consensus to restrict the use of antimicrobials in food and companion animals (3). The One Health approach takes a systems view of these complex infectious disease problems, recognizing interconnections of people and animals in their shared environments. The One Health lens encompasses the social, economic, cultural, physical, built, and political environments that can either promote or inhibit the prevention and control of these diseases. A One Health approach can identify and implement robust and meaningful solutions to improve the health and wellness of people, animals, and the environment within existing social, economic, and political contexts (12).

The current issue "*Zoonoses - a one health approach*" of "*Frontiers in Public Health*" focuses on the importance of the One Health approach in tackling complex problems such as AMR and zoonosis. The topics covered include an opinion article (Singh et al.) on the

historic developments associated with the standard definition of *zoonosis*, discrepancies in the usage of the term *zoonosis*, and suggestions for the introduction of additional terms such as *Olazoonosis*, *Akrizoonosis*, *Anakrizoonosis*, *Zoizoonosis*, *Nekrózoonosis*, and *Pidózoonosis* in the published literature. The importance of One Health approaches for zoonotic disease surveillance is highlighted by Riley et al. using zoonotic disease notifications from the period of 1996–2021 in Aboriginal and Torres Strait Islander populations in Australia.

Emerging and re-emerging viral diseases are a serious threat in Southeast Asia, and there is a need to understand the drivers of disease emergence and transmission to human and animal populations. In this issue, the application of One Health to meet this challenge in Southeast Asia has been elaborately discussed (Saba Villarroel et al.). Wet markets have proven to be a critical source of disease emergence, and Islam et al. in this issue have discussed the estimated risk factors associated with avian influenza virus (H5 and H9) contamination in live bird markets located in rural and peri-urban regions in Bangladesh.

The One Health approach is also crucial for the control and elimination of neglected tropical diseases (13). The utility of a One Health model in the detection of canine rabies cases when coupled with an integrated bite case management program in Vietnam (Ross et al.) is thus showcased in this issue. However, several issues might arise in One Health-influenced collaborations. In this issue, Suschinel et al. present the challenges in conducting an international research project on leishmaniasis in Colombia, including collaborations amongst public health institutions and dog owners.

Endemic zoonoses are responsible for a substantial burden on human and animal health, particularly in developing countries, and are deterrent to the economy (14). Preventive One Health interventions have been recommended in controlling endemic zoonoses (15). In this issue, Ayalew et al. have presented a situation assessment of zoonotic tuberculosis in Ethiopia. It is recommended to advocate for a One Health approach for the control of endemic zoonoses as the lack of knowledge on zoonoses such as brucellosis in medical professionals could result in disease misdiagnosis (Qin et al.).

Similar to zoonoses, AMR is an important One Health priority because it is a global threat to the One Health ecosystem (16). Therefore, a longitudinal One Health analysis of antimicrobial use and resistance patterns in humans and food-producing animals residing in Europe has been presented (Rahman and Hollis). The epidemiologic aspects of AMR in companion animals in the United States to inform One Health AMR programs have also been included (Sobkowich et al.). Lastly, the application of a One Health approach to understand the perceptions of different stakeholders on antimicrobial stewardship has been undertaken by identifying the associated drivers and barriers in Canada (McCubbin et al.).

In summary, the Research Topic "*Zoonoses - a one health approach*" highlights many important aspects of the One Health approach to control AMR and zoonosis.

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Zoonosis–Why we should reconsider "What's in a name?"

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1. Introduction

In 1855, Rudolph Virchow (1821–1902) used the word *zoonosis* for the first time in his famous "Handbook of Communicable Diseases" (1). The description of the word *zoonoses* or *zoonosis* is etymologically varied, although there seems to be a consensus in the published literature that *zoonosis* is a combination of two or three Greek words. The first version is that *zoonosis* is derived from two Greek words "ζῷον" (zóon—animal or living species) and "νόσος" (nósos—disease or unwell) (2). The second version uses the word "noson" in place of "nosos" but has a similar meaning (3, 4). The third version is that *zoonosis* is a combination of the words "zoo" (relating to animals or more generally to life or living things), "noso" (a person who studies disease) and "osis" (referring to a disease) (5). It is also important to note that the terms *anthroponosis* (plural *-es*; when the source is an infectious human; interhuman transfer is typical) and *sapronosis* (plural *-es*; the source is an abiotic substrate, non-living environment; interhuman transfer is exceptional) were also common for non-zoonotic human infections (6). Note the common usage of the suffix "nosis" after the stem of all the three terminologies (anthroponosis, zoonosis and sapronosis).

Independent literature on the roots and origins of medical terms describe the meaning of these Greek words as follows: zóon ('ζῷον")—animal or living thing; nósos ("νόσος")—disease, plague, anguish; osis—a suffix meaning a condition, process, activity (7). Therefore, it is highly likely that "zoon" and "nosos" are the most appropriate words in combination to form the word "zoonosis". The above suggests the etymologic meaning of *zoonosis* to be either *diseases of animals* or *the study of diseases of animals*. The usage of the words "zoon" and "nosos" is not limited to Greek. As human life was greatly influenced by animals and languages are believed to have a common ancestor, the parallel existence of these words in other languages is expected. For example, the words "Joon" (meaning a living species, or much more commonly an animal species) and "naasaaz" (meaning unwell or unrhythmic) exist in some of the Indo-Aryan languages.

2. The standard definition (1978) and the stalemate thereof

The historic legacy and diversity of the word *zoonosis* was also implicitly embedded in its definition when it was defined by the Joint WHO/FAO Expert Group on Zoonoses. In their first report in 1950 The Expert Group defined zoonosis as "those diseases which are naturally transmitted between vertebrate animals and man" (8).

In their 1958 meeting (second report), the Joint WHO/FAO Expert Group on Zoonoses noted that terms such as anthropo-zoonoses (diseases transmitted from animals to man) and zoo-anthroponoses (diseases transmitted from man to animals) had been proposed in the

published literature (9). However, it was the committee's opinion that the definition of zoonosis (as defined in the first expert group meeting) had been widely recognized and accepted, and that advocating for the use of "anthropo-zoonoses" and "zoo-anthroponoses" had many drawbacks (9). In addition, this second report also recognized the difference between disease and infection, and slightly modified the definition (Box 1) of zoonoses to be "Those diseases *and infections* which are naturally transmitted between vertebrate animals and man" (9).

In the 1966 meeting (third report) of the Joint WHO/FAO Expert Group on Zoonoses, the committee recognized that the term zoonosis is etymologically inexact and of little biological merit but found it to be useful enough to promote the prevention and control of zoonotic diseases at the human–animal interface and to provide common ground for medical and veterinary professionals (10). The committee accepted that the definition is too broad and includes situations such as diseases produced by non-infective agents (for example toxins and poisons) and infections that animals acquire from man (merely incidental infections of no public health importance) (10). The wide usage of the term *zoonosis* prevented the Committee/expert group from making any amendments to its definition. However, the Joint WHO/FAO Expert Group on Zoonoses, 1967 (10) did recommend that only those infections with a proof or strong circumstantial evidence of transmission between animals and man should be considered zoonotic diseases.

In 1978, the WHO Expert Committee on Parasitic Zoonoses (with the participation of FAO) agreed with the viewpoint of the Joint WHO/FAO Expert Group on Zoonoses, 1967 (10) and defined zoonoses as "Those diseases and infections [the agents of] which are naturally transmitted between [other] vertebrate animals and man" (11). However, the committee proposed that FAO and WHO should keep this matter under review in light of scientific developments and practical requirements (11). No change in the definition of *zoonosis* has been proposed or discussed since then by the relevant international organizations (WHO, FAO or WOAH).

3. Highlighted limitations

Important "definition" issues highlighted by scientists include the absence of clarity on whether to include or exclude zoonotic conditions such as inoculation of vertebrates (humans) by venom or toxins of reptile or fish origin, or by allergens; or diseases transmitted *via* food of animal origin (3, 4). Furthermore, the availability of sufficient evidence that demonstrates natural transmission of many accepted zoonoses has been questioned. There are demands to include unnatural opportunistic infections in immune-compromised patients by organisms of invertebrate origin (12). Unnatural (deliberate) or experimental transmission of human infectious disease agents to other vertebrates is also an issue to consider (12).

It is pertinent to note that the scientific fraternity failed to follow recommendations of the Joint WHO/FAO Expert Group on Zoonoses, 1967 (10) and the WHO Expert Committee on Parasitic Zoonoses with the participation of FAO (11) with respect to differentiating zoonotic and nonzoonotic pathogens.

4. Discrepancies in the usage and the confusion thereof

Whether intentional (for the ease of understanding and communication) or not, the World Health Organization has maintained three different versions of the definition of zoonoses:

a) A zoonosis is defined as the disease and infection naturally transmitted between people and vertebrate animals (http://www.emro.who.int/about-who/rc61/zoonotic-diseases.html).

b) A zoonosis is any disease or infection that is naturally transmissible from vertebrate animals to humans (https://www.who.int/news-room/fact-sheets/detail/zoonoses).

c) A zoonosis is an infectious disease that has jumped from a non-human animal to humans (https://www.who.int/news-room/fact-sheets/detail/zoonoses).

A recently published tripartite guide of the international organizations (FAO/WOAH/WHO) also introduces zoonoses as "diseases shared between animals–including livestock, wildlife, and pets–and people" (13).

Furthermore, multiple studies since 1967 classifying or categorizing zoonotic and non-zoonotic pathogens based on different definitions of *zoonoses* have been conducted. Jones et al. (14), Taylor et al. (15), Woolhouse and Gowtage-Sequeria (16), Singh et al. (17), Olival et al. (18) are noted examples. However, there is often a failure to recognize differences in the results produced by such studies due to different methodologic definitions of *zoonosis*. This is a barrier to research in the area of drivers of zoonoses.

Species-jumping is an inherent phenomenon of pathogens. It is believed that most of the novel human pathogens discovered, or yet to be discovered in human populations, are likely to be species-jumping pathogens from other vertebrate animals. There must be a distinction between pathogens that are regularly transmitted from non-human vertebrates to humans (e.g., rabies virus) and those that have jumped from non-human vertebrate(s) to the human population and have become adapted to human-to-human transmission (e.g., HIV, and probably now SARS-CoV-2). If the animal origin was sometime in the past but animals are no longer needed to perpetuate the cycle of transmission, can we still call these zoonoses? It is difficult to think of HIV in the 21st century as a functional zoonotic disease.

This ambiguous and non-specific definition of the term creates problems in classifying diseases. For example, there was a debate in the scientific community at the start of the COVID-19 pandemic about whether to classify COVID-19 as a zoonotic disease. Although the disease is considered to originate from wildlife, COVID-19 virus efficiently transmits between humans and does not require an animal host for maintenance. Similarly, although initially the 2009 H1N1 pandemic influenza jumped from pigs to humans, it did not require any animal species for transmission after it became established in the human population. Would this influenza virus be classified as a zoonotic pathogen? The same also holds true for the human monkeypox virus infections. A consistent and logical classification of zoonotic pathogens is essential when conducting research to characterize these pathogens and explore drivers for the emergence of zoonoses.

5. Suggested terminology and classification criteria

The word *zoonosis* is etymologically inexact, but its usage is very common and simple to follow. We suggest that international bodies (WHO/FAO/WOAH) should allow minor modification(s) in the word *zoonosis* and introduce additional terms for differentiating human infections shared among non-human animal and [other] vertebrate species (Table 1).

We propose the following terminologies to be used for different types of host-based disease categories:

Olazoonosis: Those diseases or infections [the agents of] which are naturally transmitted between non-human animals and humans. Note that this term is very broad compared to the existing definition of *zoonosis* and includes infections emanating from both vertebrate and invertebrate species. Note the usage of Greek όλα (pronunciation "óla," meaning all). Examples include rabies, echinococcosis, malaria, brucellosis and many other transgenerational or transstadial vector borne human diseases. Although infections shared between invertebrate animals and humans do not fit into the scope of the existing definition of *zoonosis*, the WHO uses this broader definition (https://www.who.int/news-room/fact-sheets/detail/zoonoses). The Center for Disease Control and Prevention, USA also defines *zoonotic diseases* (also known as zoonoses) as those caused by germs that spread between animals and people (https://www.cdc.gov/onehealth/basics/zoonotic-diseases.html). In addition, we highlight a paper on emerging infectious disease events published in *Nature* that defined zoonotic pathogens as those that originated in non-human animals (14). However, interpreting and comparing such research with that conducted using the standard definition is unwise. We argue that introducing an additional term *Olazoonosis* will be beneficial for better understanding and differentiating *Akrizoonosis* and *Anakrizoonosis*.

Akrizoonosis: Those diseases and infections [the agents of] which are naturally transmitted between [other] vertebrate animals and humans. The definition of *Akrizoonosis* is a synonym of the existing definition of *zoonosis*. Note the usage of Greek ακριβής (pronunciation "akrivís," meaning exact). Examples include rabies, echinococcosis and brucellosis. We believe that introducing the term *Akrizoonosis* would provide options for those tempted to use a broader rather than the standard definition of *Zoonosis*.

BOX 1 The historical developments in the definition of "zoonoses". Note that modification(s) adopted if any have been italicized and highlighted.

Year	Zoonosis (Definition)	References
1950	Those diseases which are naturally transmitted between vertebrate animals and man.	(8)
1958	Those diseases ***and infections*** which are naturally transmitted between vertebrate animals and man.	(9)
1966	No modification(s) suggested.	(10)
1978	Those diseases and infections ***[the agents of]*** which are naturally transmitted between ***[other]*** vertebrate animals and man.	(11)

TABLE 1 Criteria used for the classification of different zoonotic diseases.

Term	Greek usage	Transmission type	Animals involved	Examples
Olazoonosis	όλα (pronunciation "óla," meaning all)	Natural	Vertebrates and/or invertebrates	Rabies, echinococcosis, malaria and brucellosis
Akrizoonosis	ακριβής (pronunciation "akrivís," meaning exact)	Natural	Vertebrates (± invertebrates)	Brucellosis and rabies
Anakrizoonosis	ανακριβής (pronunciation "anakrivís," meaning inexact)	Natural	Only invertebrates (No vertebrate)	Non-zoonotic onchocerciasis and malaria
Akrizoonosis types				
Zoizoonosis	Zoi (pronunciation "zoí," meaning to live)	Strong circumstantial evidence of an ongoing transmission between vertebrates and humans.	Vertebrates	Brucellosis, rabies, plague, taeniosis and echinococcosis
Nekrózoonosis	νεκρός (pronunciation "Nekrós," meaning dead)	No strong evidence of an ongoing transmission between vertebrates and humans. Human-to-human transmission does not occur or is uncommon.	Vertebrates	*Trypanosoma evansi* infections, foot and mouth disease, and lumpy skin disease.
Pidózoonosis	πηδώντας (pronunciation "pidóntas," meaning jumping)	The pathogen jumps from [other] vertebrate species to humans and establishes as anthroponosis. Ongoing human-to- human transmission is very common.	Vertebrates	SARS-CoV-2, Dengue and HIV infections.

Anakrizoonosis: Those diseases and infections [the agents of] which are naturally transmitted between invertebrate animals and humans. Note the usage of Greek ανακριβής (pronunciation "anakrivís," meaning inexact). Although human–invertebrate shared diseases do not fit into the existing definition of *Zoonosis*, they are definitely different from human-specific infections (Anthroponosis). *Anakrizoonosis* includes all vector-borne infections such as non-zoonotic onchocerciasis and malaria.

6. Classification of zoonoses

We support the classification criteria and different classes of zoonotic infection(s) and disease(s) adopted by the FAO/WHO expert group (10); however, we recommend allowing usage of the etymologically exact *Akrizoonosis* in parallel to the current usage of *zoonosis*.

We also recommend that based on the frequency and temporal trends in the diseases or infections [the agents of], *akrizoonosis* (currently defined *zoonosis*) may be additionally categorized into the following:

1. *Zoizoonosis*: those diseases and infections [the agents of] which are naturally transmissible between [other] vertebrate animals and humans. In addition, there is a proof or strong circumstantial evidence of an ongoing transmission between vertebrates and humans. Note the usage of Greek "Zoi" (zoí; meaning to live). Examples include diseases such as brucellosis, rabies, plague, taeniosis and echinococcosis.
2. *Nekrózoonosis*: those diseases and infections [the agents of] which are naturally transmitted between [other] vertebrate animals and humans. However, there is no strong proof (or only weak circumstantial evidence) of an ongoing transmission between vertebrates and humans. Note the usage of Greek "νεκρός" (Nekrós; meaning dead). This term is intended for those zoonoses which are eradicated or no longer exist in vertebrate animal reservoirs. In addition, diseases with rare zoonotic incidence or presenting with only weak circumstantial evidence–such as *Trypanosoma evansi* infections, and Foot and Mouth disease–could be included within this category of zoonosis. In addition, any disease of debatable or questionable zoonotic potential (for example lumpy skin disease) may also be included.
3. *Pidózoonosis*: those diseases [the agents of] which jump from [other] vertebrate species to humans and establish as anthroponosis (human-specific pathogens). Note the usage of Greek word "πηδώντας" (pidóntas; meaning jumping). Examples include dengue, SARS-CoV-2, Dengue and HIV infections.

The Joint WHO/FAO Expert Group on Zoonoses, 1967 recognized that the classification of zoonoses is beneficial, in particular it has value for teaching and that the classification criteria should place emphasis on the epidemiology of zoonotic diseases (10). Similarly, the WHO Expert Committee on Parasitic Zoonoses (with the participation of FAO) noted that there are a large number of zoonotic diseases that demand classification for teaching purposes (11). We believe that the proposed classification perfectly follows the Expert Group guidelines and will enrich and broaden the understanding of Zoonoses by students within medical, veterinary and other related disciplines. It will also highlight the vast differences in the frequency and temporal trends in the transmission of different zoonotic pathogens. The proposed classification will complement the ongoing *reservoir host* and the *type of lifecycle* based classification criteria.

The proposed terms will be valuable for the conduct and understanding of predictive modeling and risk factor investigation studies that use objective zoonotic disease classification data (yes/no) to parameterise different statistical models to determine hotspots or drivers of disease emergence and zoonoses.

Lastly, we would also like to introduce a new term for the infections naturally transmitted among non-human vertebrates.

Therionosis: those diseases or infections [the agents of] which are naturally transmitted between nonhuman vertebrate animals. For example, neosporosis. Note the usage of the word "therion" coming from the Greek θηρ or θηρίον (meaning wild animal) in this terminology. We believe that identification of such diseases in different animal host species will help develop strategies for comprehensive control of these diseases.

We argue that etymologically exact definitions will be important for clarity and brevity in the future. Although any change in nomenclature will face difficulties in adoption and for understanding of the published literature since 1855, the lack of nomenclature differentiation between diseases naturally transmitted (or transmissible) between nonhuman animals and humans; nonhuman vertebrates and humans; and invertebrates and humans has caused miscommunication and made the scientific literature difficult to interpret for both the scientific and non-scientific communities. It is timely for international bodies (WHO, FAO and WOAH) to reconstitute the Joint WHO/FAO/WOAH Expert Group on Zoonoses and to develop guidelines on the usage of the word "zoonosis." A focus group discussion in the multidisciplinary One Health High Level Expert Panel (OHHLEP) could also be undertaken. Before being officially introduced, scientific evaluation of the adoption potential of these terms should also be conducted.

Author contributions

BS: conceptualization, definition(s), and writing—original draft. MW, PK, and ND: manuscript editing and review. All authors contributed to the article and approved the submitted version.

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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Challenges during the realization of an international research project on leishmaniasis in Colombia

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Leishmaniasis is an infectious disease that belongs to the top 10 neglected tropical diseases. It mainly affects the poor population from tropical and subtropical areas of the World, which lacks sufficient resources and means to fight against this disease. With this in mind, the European Commission has funded an international collaborative research project in which are participating various institutions from South America, North Africa and Europe. The main objective of this project is the development of a fast, less expensive, non-invasive and easy to use alternative method for leishmaniasis diagnosis in dogs, one of the main reservoirs of leishmaniasis spread to humans. In this perspective article, we present our personal insight and opinion regarding the challenges of realizing a joint international research project on leishmaniasis in Colombia, a country where leishmaniasis is endemic, as well as regarding the involvement of the Public Health institutions and the local population from this country.

KEYWORDS

public health, international cooperation, Colombia, leishmaniasis, dogs

1. Introduction

Leishmaniasis is a parasitic disease produced by the protozoa of the genus Leishmania that affects both animals and humans. It is transmitted through the bite of female sandflies, which ingest the parasite during the process of feeding with blood from a person or animal infected with the parasite, transmitting it afterwards to an uninfected animal or human being (1).

At the global level, leishmaniasis is spread mainly in the tropical and subtropical areas of the World, where the habitat of the insects that transmit the disease is mainly found. These areas include Central and South America, northern and eastern parts of Africa, eastern and southeastern Asia, the Middle East, and the Mediterranean basin area in southern Europe (2).

The global prevalence of leishmaniasis in humans is estimated to be of approximately 12 million cases, with an annual incidence rate of 1.3 million new cases and an annual death rate of approximately 70,000 people (3). Depending on the form of the disease, it is worth noting that approximately 80% of the cases of cutaneous leishmaniasis (one of the two main forms of the disease, characterized by ulcerations produced on the skin) have been reported in Colombia, Brazil, Iraq, Pakistan, Syria, Afghanistan and Algeria (4), whereas 90% of the cases of visceral leishmaniasis (the other main form of the disease, which affects the spleen, liver and bone marrow) have been reported in Brazil, Ethiopia, India, Bangladesh, Sudan and South Sudan (5).

The disease mainly affects the poor population, whereas its prevalence is higher in the rural and peri-urban areas and in jungle environment (6). Malnutrition, population displacement, housing with poor living conditions, lack of resources and weak immune system are other important factors that favor the transmission of this disease (7). Leishmaniasis is also linked to environmental changes such as deforestation, urbanization, construction of dams and irrigation systems (8).

Thus, the most exposed people to this disease belong to the poorest strata of the population, with inadequate housing conditions and low socio-economic position in the society, for which the access to treatment can be furthermore prohibitive, besides of being challenging (9). If left untreated, the cutaneous form of leishmaniasis can cause permanent signs such as deformity and disfigurement, while the visceral form can cause death in more than 90% of the untreated cases (10).

Early and accurate diagnosis of this disease are essential for prescribing an adequate treatment and preventing the further transmission of the disease, as well as for improving the quality of life of the patients. Visual inspection of the patient is the first step in identifying certain signs compatible with Leishmaniasis (sores, weight loss, fever, enlargement of spleen and liver), but the clinical manifestations of the disease are not specific only to leishmaniasis, whereas some patients can have a silent infection without presenting symptoms or signs (11). Confirmatory tests, which include different parasitological, serological and molecular techniques (e.g., ELISA, indirect fluorescence antibody test, rapid immunochromatographic test, PCR), are expensive and time-consuming, and are not always available in routine daily practice in the disfavored areas where the disease is predominantly spread (12).

In light of these considerations, research is being performed for developing alternative diagnostic procedures for leishmaniasis, which should be less expensive, provide rapid results and could be performed on-site with minimal preparation. Such diagnostic procedures need to be extended also to animals, mainly dogs, which may be considered potential reservoirs for leishmaniasis transmission to humans (13), although their roles as reservoirs need to be adequately demonstrated (14, 15). Poverty is highly correlated with cohabitation with a high number of mongrel dogs, which is an important risk factor that further favors the spreads of the disease to the vulnerable population (16).

In this regard, we have performed various field activities in Colombia in the framework of an international research project that is aiming the development of a volatile test for non-invasive, easy and fast diagnosis of leishmaniasis in dogs, based on the analysis of their exhaled breath and of the volatiles released by their hair, an approach that was previously assessed to detect dogs with visceral leishmaniasis in Brazil (17). Our study is justified in the context of the epidemiologic control fragility of canine leishmaniasis in Colombia, which occupies the second place after Brazil in the number of cases of canine leishmaniasis in the Latin America region (15). The cases of human leishmaniasis are also seen as a health risk problem in Colombia, especially for the adult males, as they become infected when they enter the vector's biotopes to tap natural resources, or into the jungle for illicit crops culture, guerrilla-type activity or military actions to fight against the anterior ones, where the soldiers are accompanied by military dogs trained to detect landmines, which are exposed to sandflies infected with the leishmania parasite and are prone to acquire the disease (15, 18).

In this article we wish to present our viewpoint regarding the attitude and involvement of the Public Health institutions and dog owners from endemic zones of leishmaniasis in Colombia that we contacted for conducting our research, as well as the challenges that we needed to overcome.

2. Research project and activity performed

Research activities were performed in Colombia in the framework of the international research project CANLEISH funded by the European Commission (19), which is aiming the investigation and development of an alternative method for the diagnosis of leishmaniasis in dogs based on volatile samples analysis. The execution of this project was approved by the Committee for Ethics and Environmental Impact in Research of University of Pamplona, Colombia (Approval Certificate No. 002 from April 14th, 2021).

Various regions from Colombia affected by outbreaks of canine leishmaniasis, as well as isolated cases, were identified from the information published by the National Institute of Health from Colombia. Both cutaneous and visceral forms of canine leishmaniasis were considered, for better encompassing the different manifestations of this disease.

Leishmaniasis diagnosis at the dogs included in this study followed standard procedures. In the case of the dogs with skin lesions compatible with cutaneous leishmaniasis, smear samples were taken from the skin lesions for microscopic examination and parasite identification. Biopsies were also taken from the lesion in order to perform PCR analysis. In the case of the dogs suspected for visceral leishmaniasis, approximately 3 ml of blood samples were taken from the cranial area of the forepaw of the animal in order to apply the rapid immunochromatographic test for the rK39 antigen. Biopsies were moreover taken, employing the thin needle aspiration procedure, from the popliteal lymph nodes, located in the caudal area of the hind paw of the animal, when these nodes presented swelling effect. Before taking the biopsy, the animals were tranquilized using Tranquilan® (Acepromazine) at a commercial dose (1 mg per kg of body weight, IV). From the sacrificed animals, spleen biopsies were taken after euthanasia by thin fine needle aspiration biopsy. Euthanasia was performed by administration of Eutanex® (sodium pentobarbital 390 mg/ml, sodium diphenylhydantoin 50 mg/ml) at a commercial dose (1 ml

per each 5 kg of body weight, IV). The biopsies were examined by PCR analysis.

Specific field work related with the realization of the CANLEISH project consisted in the collection of different samples from the dogs for volatile analysis. A specific protocol was developed for this aim. Breath samples were taken by introducing dog's head inside an equine nebulization mask, which was adjusted such that to prevent the escape of animal's head (Figure 1). The volatile compounds emitted by dogs through breathing were acquired in Tenax sorbent tubes by pumping, as previously reported (20). Nervous dogs were tranquilized with Tranquilan® at a commercial dose (1 mg per kg of body weight, IV) before performing this process. Additionally, approximately 10 mg of hair was cut near the lesion and stored in 125 ml wide mouth specimen jars. At a later stage, the volatiles released by hair were transferred to Tenax sorbent tubes through pumping, inside a biosafety cabinet for avoiding external contamination, in a similar way as reported for the acquisition of the volatiles emitted by feces (20). The volatiles stored in the Tenax tubes are currently analyzed employing various volatolomic techniques, and the results obtained will be published elsewhere.

FIGURE 1
Mask used during breath sampling.

3. Involved participants

3.1. Researchers

Following the specific rules of the project in whose framework the activities of this study were performed, which is based on the exchange of researchers between the participating institutions, researchers from three different countries took part at the field work. Researchers from Colombia, the country where the animals were selected and sampled, included veterinary, parasitology and pathology professionals, with experience in different diagnostic methods for infectious disease, and an electronic engineer. Researchers from veterinary institutions from two European countries, Estonia and Romania, have also participated. The European team included veterinary medicine students and a senior researcher with experience in sensor systems development for volatile samples analysis.

3.2. Public authority

The aim of this study was communicated to representatives of the regional Public Health Authorities and Mayor's Offices from the Departments[1] of Colombia where the animals were selected for the study. The participation of the Public Authorities was in agreement with the operative procedures outlined by the Health and Social Protection Ministry of Colombia (21).

In the Department of Huila, where dogs with visceral leishmaniasis were sampled, representatives from the Departmental Health Secretary of Huila accompanied the researchers during their field work activities, and provided them assistance in detecting possible cases of canine leishmaniasis and dogs' sampling. The Public Authority staff that participated at the field work included three veterinarians, one biologist and 14 technicians from the Public Health Department, as well as six municipal employees.

In the Department of Norte de Santander, where dogs with cutaneous leishmaniasis were sampled, it was instead medical staff from the Public Health Services of various villages where animals were sampled who provided the same assistance to the researchers from the project.

3.3. Dog owners

Dog owners were inhabitants from rural and peri-urban areas with deficiencies in public services and a high level of poverty, lacking of basic sanitation or hygiene facilities. They were previously informed about the aim of the study and the day of dogs' sampling by the representatives of the local Public Health Authorities. Those who accepted their dogs to be included in the study received, in the day of sampling, detailed information from the researchers about the project, regarding both the objective of the study and the sampling procedure. They were also informed about researchers' obligation to communicate the results of the official standard tests for leishmaniasis realized in parallel with this study to the public health authorities in case of a positive result. The dogs were included in the study and sampled only after their owners signed the informed consent.

1 Administrative territorial division in Colombia.

FIGURE 2
Researchers, public health staff, and local community participating at dogs' sampling.

4. Discussion

4.1. Research framework

With the objective to provide useful means for community-based interventions aimed at the prevention and control of the spread of leishmaniasis, a disease that belongs to the top 10 neglected tropical diseases as from Pan American Health Organization (22), the European Commission funded an international collaborative research project in which different institutions from Colombia, Europe and North Africa are participating (19).

Although leishmaniasis is not generally present in most of the European countries, where its prevalence is mainly limited to the Mediterranean basin area, the adoption of dogs from southern Europe to other European countries led to the expansion of the number of reported cases in other European countries such as Great Britain or Germany (23). The European veterinarians are not however much exposed to canine leishmaniasis cases, therefore their ability to accurately diagnose or recognize the signs of this disease is very limited.

Actually, in accordance with European legislation, Directive 2003/99/EC of the European Parliament and of the Council amended by Council Directive 2013/20/EU, leishmaniasis is not included on the list of parasitic zoonoses that must be monitored within the epidemiological surveillance in Europe. Knowing the challenges of the situations when this disease occurs, the international collaboration within this project is helping researchers, authorities and the population from Europe to be better informed and to be able to manage this zoonosis and the problems faced by the public health also in Europe. In this regard, the research secondments performed in the framework of the CANLEISH project by staff from veterinary institutions from Estonia and Romania in Colombia, a country where leishmaniasis is endemic, represents an unvaluable opportunity for them for changing experiences in the diagnosis of this disease with their more experienced Colombian colleagues.

On the other hand, this project offered also a unique opportunity for the Colombian authorities, veterinarians and researchers to meet and collaborate with their counterparts from Europe during the realization of joint research and field activities in Colombia. The importance of this collaboration was reflected by the reception of the researchers in the Mayoralty of various towns from Colombia, and by the highlights published by local newspapers from Colombia regarding the project (24, 25).

In this perspective paper we do not however aim to present the scientific results of this study, which will be published elsewhere after the volatile samples collected from dogs during the field work performed in Colombia will be exhaustively investigated. Instead, we are focusing here in presenting our personal insight and opinion regarding the experiences and challenges of realizing a joint international research project on leishmaniasis in Colombia.

4.2. Public health authority involvement

Representatives of the public health authorities accompanied the researchers on the field, acting as an intermediary between the local population and the researchers. Their level of involvement and interest for the project were slightly different, depending on the

spread of the disease and its effects in the specific territories where dogs were sampled.

In Colombia, human and canine visceral leishmaniasis are restricted to two major transmission foci, the Caribbean coast and middle Magdalena River Valley (26). The city of Neiva, capital of the Department of Huila, situated on the middle Magdalena River Valley, had suffered not long ago outbreaks of visceral leishmaniasis that produced the death of a baby child that acquired the disease from an infected dog (27). As a consequence, the Departmental Health Secretary of Huila started a very intensive campaign in the peri-urban area of Neiva aiming to control the disease and stop its further spread, to which it dedicates a team of 25 public servants and economical recourses of more than 45,000 USD / month for fighting against both leishmaniasis and dengue (another vector-borne infectious disease largely spread in Colombia). One of the measures taken was the identification of the dogs affected by canine leishmaniasis. Our project was perfectly matching their objective, therefore in Neiva the health authorities at the departmental level supported the work of our researchers. The field work was done in parallel by the staff of the Public Health authority, which were collecting blood samples from all the dogs from the affected areas for performing the rapid immunochromatographic test against rK39 antigen, and by our researchers. The staff of the Public Health authority from Neiva has also applied euthanasia to the dogs diagnosed with leishmaniasis.

In the Department of Norte de Santander, where the cases of leishmaniasis are only sporadic, the situation was different. The Public Health authority at departmental level didn't get actively involved in our study, but there were rather the representatives of the local health services from the villages where isolated cases of cutaneous canine leishmaniasis had occurred who showed higher interest in our project, and assisted us in identifying possible cases of canine leishmaniasis and accompanied us in our field work. Given the more limited financial resources of the health services acting at the local level, the diagnosis of canine leishmaniasis was realized in the Laboratory of Biomedical Sciences of University of Pamplona by microscopy analysis and PCR.

4.3. Population involvement

In Colombia, in conformity with the current legislation regarding zoonotic diseases (Decree 2257 from 1986), in addition to the mandatory notification of a zoonotic disease, article 49 imposes the elimination by the health authorities of the animals that present a zoonotic disease. Therefore, every diagnosed case of canine leishmaniasis needs to be communicated to the health services for taking appropriate measures. This legal provision creates reticence among the population when participating to a study that could lead to the diagnosis of a zoonotic disease in their pet, because of the very close link that is normally created between humans and their pets.

Due of this reason, in the Department of Norte de Santander, where the cases of canine leishmaniasis are only isolated, it happened that some dog owners, aware about our study and the day we were coming for dogs' sampling, decided to take their dogs away from their home and did not show up during all the day. Nevertheless, other people more aware about the gravity of this disease and its consequences, traveled even very long distances from their difficultly reachable living places for bringing to us their dogs for testing and sampling.

Instead, in the Department of Huila, the occurrence of such a tragical case as it was the loss of a baby child's life, made all the community aware of the transcendental importance of timely detection of the dogs infected with leishmaniasis. For this reason, the whole community was prone to participate in our study. However, as the sampling was done in the peri-urban area of the city of Neiva, along an illegally built settlement, it was necessary that the representatives of the Public Health Service, who already knew and had contact with the local people, explained them previously the scope of our presence there in order to avoid unpleasant conflictual or other kind of dangerous situations. They became thus very collaborative and participative, and when we arrived there for sampling, the leader of the community announced over the megaphone the local people to bring their dogs at the established meeting point for sampling (Figure 2).

4.4. Researchers' challenges

The realization of such a research project that encompassed field work in remote and difficulty accessible communities from a South American country like Colombia implied important challenges for the involved researchers, which ranged from difficult access through roads not properly prepared for car traffic and not lacking of danger, up to people reticence in front of the presence of unknown persons. The great help and assistance received from the staff of the local health services, who accompanied the project researchers on the field and took care of the proper contact with the local people, was essential for the successful realization of this part of the study, which consisted in sampling the volatiles emitted by suspicious dogs for leishmaniasis through breathing and hair. The enthusiasm and no renunciation of the researchers participating in the study were also very important for achieving our objective. Special appreciation in this regard deserves the European researchers from Estonia and Romania, who were not used with the working conditions and customs from a South American country.

Before finishing this perspective point of view, we wish to specially remark the great involvement of the civil servants of the legal authorities from Colombia (Public Health institutions and Mayoralties) and of the local people that, although lacking minimal living conditions, treated us with their highest availability and hospitality. They didn't hesitate any moment to bring us chairs from their houses to sit, or to share with us the scarce food and drinks they had, which was highly appreciated in the torrid tropical days from the Tropics, and in the absence of food and beverage stores. The memories gathered during this joint research project will be never forgotten by the researchers that had the opportunity to live such an amazing experience.

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 Committee for Ethics and Environmental Impact in Research of University of Pamplona, Colombia (Approval Certificate No. 002 from April 14th, 2021). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

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

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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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Prevalence and risk factors for avian influenza virus (H5 and H9) contamination in peri-urban and rural live bird markets in Bangladesh

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Avian influenza viruses (AIV) have been frequently detected in live bird markets (LBMs) around the world, primarily in urban areas, and have the ability to spillover to other species, including humans. Despite frequent detection of AIV in urban LBMs, the contamination of AIV on environmental surfaces in rural and peri-urban LBMs in Bangladesh is poorly documented. Therefore, we conducted this study to determine the prevalence of AIV subtypes within a subset of peri-urban and rural LBMs in Bangladesh and to further identify associated risk factors. Between 2017 and 2018, we collected faecal and offal samples from 200 stalls in 63 LBMs across four sub-districts. We tested the samples for the AIV matrix gene (M-gene) followed by H5, H7, and H9 subtypes using real-time reverse transcriptase-polymerase chain reaction (rRT-PCR). We performed a descriptive analysis of market cleanliness and sanitation practices in order to further elucidate the relationship between LBM biosecurity and AIV subtypes by species, sample types, and landscape. Subsequently, we conducted a univariate analysis and a generalized linear mixed model (GLMM) to determine the risk factors associated with AIV contamination at individual stalls within LBMs. Our findings indicate that practices related to hygiene and the circulation of AIV significantly differed between rural and peri-urban live bird markets. 42.5% (95% CI: 35.56–49.67) of stalls were positive for AIV. A/H5, A/H9, and A HA/Untyped were detected in 10.5% (95% CI: 6.62–15.60), 9% (95% CI: 5.42–13.85), and 24.0% (95% CI: 18.26–30.53) of stalls respectively, with no detection of A/H7. Significantly higher levels of AIV were found in the Sonali chicken strain compared to the exotic broiler, and in offal samples compared to fecal samples. In the GLMM analysis, we identified several significant risk factors associated with AIV contamination in LBMs at the stall level. These include: landscape (AOR: 3.02; 95% CI: 1.18–7.72), the number of chicken breeds present (AOR: 2.4; 95% CI: 1.01–5.67), source of birds (AOR: 2.35; 95% CI: 1.0–5.53), separation of sick birds (AOR: 3.04; 95% CI: 1.34–6.92), disposal of waste/dead birds (AOR: 3.16; 95% CI: 1.41–7.05), cleaning agent (AOR: 5.99; 95%

CI: 2.26–15.82), access of dogs (AOR: 2.52; 95% CI: 1.12–5.7), wild birds observed on site (AOR: 2.31; 95% CI: 1.01–5.3). The study further revealed a substantial prevalence of AIV with H5 and H9 subtypes in peri-urban and rural LBMs. The inadequate biosecurity measures at poultry stalls in Bangladesh increase the risk of AIV transmission from poultry to humans. To prevent the spread of AIV to humans and wild birds, we suggest implementing regular surveillance at live bird markets and enhancing biosecurity practices in peri-urban and rural areas in Bangladesh.

KEYWORDS

avian influenza virus, live bird markets, landscape, environmental contamination, biosecurity practices, risk factors, zoonoses, Bangladesh

1. Introduction

Avian influenza viruses (AIVs) are zoonotic viruses that can infect domestic and wild bird species, along with a variety of other animals (1). Multiple subtypes of low pathogenic avian influenza (LPAI) viruses and highly pathogenic avian influenza (HPAI) have been detected from live bird markets (LBMs) and farms around Bangladesh, with H9N2 and H5N1 being the most prevalent (2–4). H5N1 and H9N2 are mostly endemic to countries in Southeast Asia, such as Bangladesh (5–7). Over 585 influenza outbreaks have been recorded in poultry in Bangladesh (4). AIVs can spillover to humans from the poultry, often presenting with severe clinical outcomes. In 1997, the H5N1 virus infected 18 people in Hong Kong, causing six fatalities. Those were the first human deaths associated with the virus (8). In Bangladesh, eight human cases of H5N1 have been detected between the years of 2008 and 2022, one of which resulted in fatality (9). Three incidences of human infection with H9N2 viruses have been reported in Bangladesh, with the most recent case involving a poultry market worker who was in contact with sick birds (6). Evidence of spillover from poultry to humans raises substantial concerns about occupational exposure to AIVs in LBMs. In addition to poultry, there have been occasional reports of spillover to house crows and evidence of AIV in captive birds at zoos and parks (10–13). These reports raise additional concerns about the potential sources of spillover to humans and implications for wildlife health.

LBMs are common sites for poultry trading, selling, and processing in Asia (14, 15). The birds are sourced from multiple locations and hoarded into densely packed cages, often with more than one breed or species in the same enclosure. It is common practice for the vendors to slaughter the birds on site and leave the offal and poultry remains in the stall (16). Moreover, LBM biosecurity is generally poor in Bangladesh, and not practiced in accordance with the guidelines recommended by the Food and Agriculture Organization (FAO) to reduce the risk of virus circulation (17). For example, one study in Bangladesh showed that LBM workers who did not follow proper biosecurity practices during daily activities, such as feeding poultry, cleaning feces from pens, and handling sick poultry were at higher risk of exposure to the virus (18). Similar findings have been reported in other countries, whereby a number of additional studies have observed risk factors associated with AIV contamination at LBMs (19, 20). As a potential hotspot for AIV infection (17, 21, 22), LBMs are in urgent need of biosecurity improvements as well as enhanced behavioral and biological surveillance.

The population of Bangladesh has increased rapidly over the past 20 years (23), resulting in a greater demand for food and intense competition for resources. This growing demand exists in urban areas as well as in peri-urban and rural areas. To meet this demand, private and governmental investment has increased to raise commercial production of protein (24). As a result, Bangladesh's production of meat has doubled over the last 10 years (25). About one-third of the country's protein comes from livestock and other animal products (26). In previous years, most rural households raised poultry in their backyard to support protein consumption (27). However, with the population increase, the dependency has shifted from backyard poultry to commercial poultry in both peri-urban and rural areas.

As more people turn to LBMs as a source of protein in Bangladesh (28), robust risk characterization is essential to inform targeted public health interventions at this interface. However, most studies about the risk of AIV biosecurity are conducted in urban areas and communities (7, 29–32). The risk of AIV spillover in peri-urban and rural areas remains poorly understood. To that end, we conducted a cross-sectional study on LBMs to explore the diversity and prevalence of AIV and their associated risk factors in peri-urban and rural areas in Bangladesh.

2. Methodology

2.1. Study design, site selection

Bangladesh is subdivided into an administrative hierarchy as follows: division > district > sub-district (upazilla) > union > village (33). We conducted this study in upazillas, unions, and village settings. We conducted a cross-sectional study among 63 LBMs consisting of 25 from Savar and 21 from Dhamrai in Dhaka district, 13 from Fulbaria in Mymensingh, and four from Pabna Sadar in Pabna district – covering both peri-urban and rural areas (Figure 1). We enrolled 2 to 5 vendors from each LBM based on the market size and landscape gradient, such as whether the market is in a peri-urban or rural settings. We enrolled a total of 200 vendors from 63 LBMs. We used a purposive sampling strategy, and only enlisted vendors who agreed and consented to participate in our study (34). Upon receiving consent, we conducted a behavioral risk questionnaire with each vendor, followed by biological sample collection from their stall or workspace.

In our study, we defined an LBM as a facility with a physical structure where vendors sell live poultry. Birds are slaughtered and sold on-site and typically remain at the market until they are sold. A vendor is a shop owner or stall keeper who buys poultry from farms or middleman to sell to other vendors or directly to consumers. Stalls

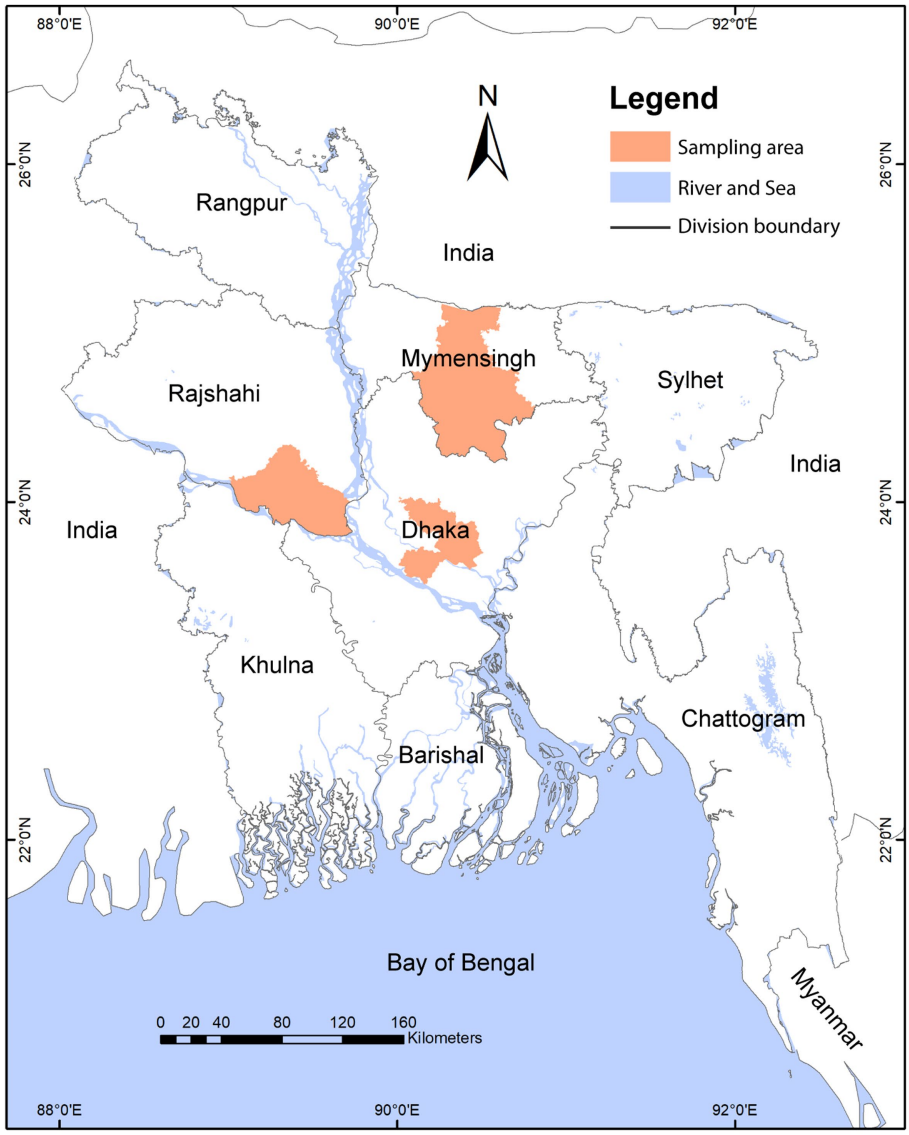

FIGURE 1
Map showing the study sites. Colored regions indicates the sampling districts of the study.

are small places within the LBM, usually owned or leased by a vendor (the shop owner) to keep, process, and sell poultry.

In Bangladesh, LBMs are regulated by different authorities, including government and private. At the peri-urban level, the LBMs are monitored and controlled by local governments (such as municipalities) or privately. In the case of rural areas, it is mainly regulated by the local government (union porishod) (35). In some cases, specific market-based associations play a vital role in the LBM operation. There are 12, 16, 13, and 10 unions under the Savar., Dhamrai, Fulbaria, and Pabna Sadar subdistrict, respectively (Figure 1).

2.2. Biological specimens and biosecurity practices data collection at the stall level

We took samples from two strains of chickens: exotic white broilers and golden colored, Sonali birds. Sonali is a crossbreed between the Rhode Island Red (RIR) cocks and Fayoumi hens (36). These extotic broilers and cross-bred Sonali chicken are sold as meat types in the LBM. We collected freshly deposited feces from the stall and offal from freshly slaughtered birds'. We obtained 2–4 fecal or offal swab samples from each stall and made them into a fecal and offal pool separately. If any dead or sick birds were available at the time of sampling, we also took pooled oropharyngeal and cloacal swab samples. We recorded all bird species present in the stall at the time of sample collection, based on observation. The swab samples were kept in a 3.6 ml cryovial, or 10 ml falcon tube containing 3 ml viral transport media (VTM) and placed in a liquid nitrogen container (−196°C). In the Laboratory, we stored the samples at −80°C in the freezer until further processing. During sample collection, the team wore appropriate personnel protective equipment like gloves and N95 masks. We prepared and pretested a questionnaire to collect data on biosafety and biosecurity practices at the stall level of LBMs. We administered the questionnaires to consenting vendors or workers through a face-to-face interview.

2.3. Ethical approval

The study protocol was approved by the Institutional Ethics Committee of the Institute of Epidemiology Disease Control and Research (IEDCR/IRB/2015/04), Chattogram Veterinary and Animal Sciences University-Animal Experimentation Ethics Committee (protocol: CVASU/Dir (R&E) AEEC/2015/751).

2.4. Laboratory testing

Following the manufacturer's instructions, we extracted RNA using the magnetic bead-based RNA isolation technique in a KingFisher Flex 96-well robot using the MagMAXTM-96 AI/ND Viral RNA Isolation Kit (Applied BiosystemsTM, San Francisco, CA). We tested the pooled fecal and offal samples from each stall separately for the presence of the AIV viral Matrix (M) gene. We evaluated the swab samples using a real-time reverse transcription PCR detection kit and fluorescent TaqMan probes to type and subtype influenza viruses (37, 38). We used primers and probes specific to the matrix gene to detect influenza A viruses. We employed H5, H7, and H9 hemagglutinin gene-specific primers and probes to detect H5, H7, and H9 subtypes in influenza A virus-positive samples (37, 39). The samples that tested positive for AIV RNA (M-gene) but negative for H5, H7, and H9 were classified as HA Untyped.

2.5. Statistical analysis

We summarized the characteristics of biosecurity practices from the questionnaire using descriptive analyses. We then determined the prevalence of AIV subtypes at the level of stall, LBM, chicken strains, and sample category along with 95% confidence intervals (CIs) and visualized them using graphical analysis. We considered a stall AIV positive if the fecal or offal sample was positive for any of the aforementioned subtypes. In addition, we labelled an LBM as AIV positive if at least one stall sample was positive for AIV by marking each stall as positive or negative for AIV and its subtype in LBMs containing multiple stall samples (40, 41). We performed univariable and multivariable risk factors analysis at the stall level. We considered a sample positive for the binary outcome variable if it was found positive either for A/H5, A/H9, or A/HA untyped in the laboratory test. We performed Pearson's chi-square test (42) to find the bio-security practices significantly associated with AIV. Factors associated with AIV with a value of p <=0.05 in univariate analysis were selected for multivariable analyses. We then used a generalized linear mixed model (GLMM) (43), accounting for clustering by LBM, to estimate adjusted odds ratios. We considered the value of p <=0.05 statistically significant in the final multivariable analysis. We calculated model χ^2 to measure model fitness for the GLMM. We performed all statistical analyses using *R* (44). We used "lme4" and "tidyverse" packages for the analysis in *R* software. We created the maps using ArcGIS v10.4.1 (ESRI, Redlands, CA, United States). The shape file was collected from freely available DIVA-GIS.[1]

1 https://www.diva-gis.org/gdata

3. Results

3.1. Hygienic status and physiographic characteristics of the studied LBMs across landscapes

We conducted this study on two different types of landscapes: 92 peri-urban LBMs and 108 rural LBMs. We noted a number of differences in the physiographic characteristics and hygienic systems between the peri-urban and rural markets. We found that the majority of vendors kept multiple strains of chicken (56.5%; 95% CI: 49.3–63.5), but we did not detect a significant difference between peri-urban and rural LMBs. Only 17.5% of the stalls in our study had ducks, although the proportion was significantly ($p = 0.04$) higher at stalls in peri-urban LBMs (87.96%; 95% CI: 80.3–93.4). Concrete flooring was more common in peri-urban markets (56.52%; 95% CI: 45.8–66.8), and a mud floor was more common in rural markets (64.81%; 95% CI: 55.0–73.8). 65.5% of all vendors kept their poultry on the floor compared to the cage. We detected a signigicant difference in the ($p < 0.01$) use of bamboo to create a stall boundary compared to brick, in rural LBMs (65.74%; 95% CI: 56.0–74.6). 66.3% of stalls (95% CI: 55.7–75.8) of peri-urban LBMs collected their birds from middlemen rather than commercial farms, and the difference was significant ($p = 0.01$) compared to rural LBMs. Most stalls had no unsold birds that stayed overnight at the shop (57.5; 95% CI: 50.3–64.4), but we did not observe a difference by landscape. The number of peri-urban markets with a bird death in the 7 days prior to sampling was significant (36.96%; 95% CI: 27.1–47.7) ($p = 0.05$) compared to rural markets. Running water supply was more common in peri-urban areas (60.87%; 95% CI: 50.1–70.9), and the percentage of stalls with no drainage system (68.5%; 95% CI: 61.6–74.9) and no electricity (62.04%; 95% CI: 52.2–71.2) was higher in the rural LBMs. Wild birds were more commonly observed at peri-urban stalls (63.04%; 95% CI: 52.3–72.9). Most of the peri-urban vendors (69.57%; 95% CI: 59.1–78.7) kept their stalls open daily, which was notably ($p < 0.01$) higher than rural vendors (42.59%; 95% CI: 33.1–52.5; Table 1).

3.2. Prevalence of AIV and its subtypes by different factors

3.2.1. Prevalence of AIV subtype at the market and stall level

We collected samples from a total of 63 LBMs during our study period. Of the 63 markets, 52 (82.54%; 95% CI: 70.90–90.95) were positive for the AIV M-gene. Overall, 23.81% (95% CI: 13.98–36.21) of markets tested positive for subtype A/H5, 22.22% (95% CI: 12.72–34.46) contained A/H9, and 58.73% (95% CI: 45.62–70.99) tested positive for HA/untyped. We recorded two instances of co-contamination with subtypes A/H5 and A/H9 at two of the LBMs. The spatial distribution of AIV subtype circulation for all sub-districst is shown in Figure 2.

The prevalence of the AIV M-gene was 42.5% (95% CI: 35.56–49.67) at the stall level. A/H5 and A/H9 positive samples were found in 10.5% (95% CI: 6.62–15.60) and 9% (95% CI: 5.42–13.85)

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TABLE 1 Frequency of physiographic and hygienic status of the studied poultry markets in peri-urban and rural LBMs.

Factors	Peri-urban *n* =92	Rural *n* =108	Total *n* =200	*p* value
	Percentage (95% CI)	Percentage (95% CI)	Percentage (95% CI)	
Chicken strain				
Broiler	55.43 (44.7–65.8)	67.59 (57.9–76.3)	62.0 (54.9–68.8)	0.11
Sonali	44.57 (34.2–55.3)	32.41 (23.7–42.1)	38.0 (31.3–45.1)	
Number of chicken strain keeping				
multiple	61.96 (51.2–71.9)	51.85 (42–61.6)	56.5 (49.3–63.5)	0.20
single	38.04 (28.1–48.8)	48.15 (38.4–58)	43.5 (36.5–50.7)	
Duck present at stall				
No	76.09 (66.1–84.4)	87.96 (80.3–93.4)	82.5 (76.5–87.5)	0.04
Yes	23.91 (15.6–33.9)	12.04 (6.6–19.7)	17.5 (12.5–23.5)	
Flooring system				
Concrete	56.52 (45.8–66.8)	35.19 (26.2–45)	45.0 (38–52.2)	<0.01
Mud	43.48 (33.2–54.2)	64.81 (55.0–73.8)	55.0 (47.8–62)	
Birds location at the stall				
Cage	36.96 (27.1–47.7)	32.41 (23.7–42.1)	34.5 (27.9–41.5)	0.60
Floor	63.04 (52.3–72.9)	67.59 (57.9–76.3)	65.5 (58.5–72.1)	
Boundary made of				
Bamboo	42.39 (32.2–53.1)	65.74 (56–74.6)	55.0 (47.8–62)	<0.01
Brick	57.61 (46.9–67.9)	34.26 (25.4–44)	45.0 (38–52.2)	
Source of birds				
Farm	33.70 (24.2–44.3)	53.70 (43.9–63.4)	44.5 (37.5–51.7)	0.01
Middleman	66.30 (55.7–75.8)	46.30 (36.7–56.2)	55.5 (48.3–62.5)	
Remain unsold overnight at the stall				
No	56.52 (45.8–66.8)	58.33 (48.5–67.8)	57.5 (50.3–64.4)	0.91
Yes	43.48 (33.2–54.2)	41.67 (32.3–51.6)	42.5 (35.6–49.7)	
Separate sick birds				
No	58.70 (48–68.9)	46.30 (36.7–56.2)	52.0 (44.8–59.1)	0.11
Yes	41.30 (31.1–52.1)	53.70 (43.9–63.4)	48.0 (40.9–55.2)	
The bird died in the last seven days at the stall				
No	63.04 (52.3–72.9)	76.85 (67.8–84.4)	70.5 (63.7–76.7)	0.05
Yes	36.96 (27.1–47.7)	23.15 (15.6–32.3)	29.5 (23.3–36.3)	
Disposal of offal and dead birds				
Burry/Dustbin	46.74 (36.3–57.4)	46.30 (36.7–56.2)	46.5 (39.4–53.7)	>0.99
Throw away	53.26 (42.6–63.7)	53.70 (43.9–63.4)	53.5 (46.3–60.6)	
Cleaning agent				
Detergent	39.13 (29.1–49.9)	25.93 (18–35.3)	32.0 (25.6–39)	0.07
Water only	60.87 (50.1–70.9)	74.07 (64.8–82)	68.0 (61.1–74.4)	
Running water supply at LBM/stall				
No	39.13 (29.1–49.9)	95.37 (89.5–98.5)	69.5 (62.6–75.8)	<0.01
Yes	60.87 (50.1–70.9)	4.63 (1.5–10.5)	30.5 (24.2–37.4)	
Are the worker/owner drink the same water				
No	86.96 (78.3–93.1)	85.19 (77.1–91.3)	86.0 (80.4–90.5)	0.88
Yes	13.04 (6.9–21.7)	14.81 (8.7–22.9)	14.0 (9.5–19.6)	

(Continued)

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TABLE 1 (Continued)

Factors	Peri-urban *n* =92	Rural *n* =108	Total *n* =200	*p* value
	Percentage (95% CI)	Percentage (95% CI)	Percentage (95% CI)	
Washing hands with soap				
No	47.83 (37.3–58.5)	55.56 (45.7–65.1)	52.0 (44.8–59.1)	0.34
Yes	52.17 (41.5–62.7)	44.44 (34.9–54.3)	48.0 (40.9–55.2)	
Dedicated cloth to work in the stall				
No	51.09 (40.4–61.7)	59.26 (49.4–68.6)	55.5 (48.3–62.5)	0.31
Yes	48.91 (38.3–59.6)	40.74 (31.4–50.6)	44.5 (37.5–51.7)	
Use of mask				
No	85.87 (77.1–92.3)	93.52 (87.1–97.4)	90.0 (85–93.8)	0.12
yes	14.13 (7.7–23)	6.48 (2.7–12.9)	10.0 (6.2–15)	
Drainage system				
No	36.96 (27.1–47.7)	95.37 (89.5–98.5)	68.5 (61.6–74.9)	<0.01
Yes	63.04 (52.3–72.9)	4.63 (1.5–10.5)	31.5 (25.1–38.4)	
Electricity at stall				
No	13.04 (6.9–21.7)	62.04 (52.2–71.2)	39.5 (32.7–46.6)	<0.01
Yes	86.96 (78.3–93.1)	37.96 (28.8–47.8)	60.5 (53.4–67.3)	
Closing day of the stall				
No	69.57 (59.1–78.7)	42.59 (33.1–52.5)	55.0 (47.8–62)	<0.01
Yes	30.43 (21.3–40.9)	57.41 (47.5–66.9)	45.0 (38–52.2)	
Access to rodents at the stall				
No	50.0 (39.4–60.6)	48.15 (38.4–58)	49.0 (41.9–56.2)	0.91
Yes	50.0 (39.4–60.6)	51.85 (42–61.6)	51.0 (43.9–58.1)	
Street dog access at stall				
No	47.83 (37.3–58.5)	50.0 (40.2–59.8)	49.0 (41.9–56.2)	0.87
Yes	52.17 (41.5–62.7)	50.0 (40.2–59.8)	51.0 (43.9–58.1)	
Observe wild birds (nuisance birds) at LBM/stall				
No	36.96 (27.1–47.7)	64.81 (55–73.8)	52.0 (44.8–59.1)	<0.01
Yes	63.04 (52.3–72.9)	35.19 (26.2–45)	48.0 (40.9–55.2)	

of stalls, respectively. We detected A/HA untyped in 24.0% (95% CI: 18.26–30.53) stalls. At the sub-district level, we found a higher prevalence for subtype H5 (46.15%; 95% CI: 19.22–74.87) in Pabna Sadar. The sub-district Dhamrai had the highest detection of subtype A/H9 (15.15%; 95% CI: 7.51–26.10), while samples from Savar had the highest detection of A/HA untyped (32.18%; 95% CI: 22.56–43.06). We did not detect subtype A/H7 in any of our samples (Figure 2).

3.2.2. Prevalence of AIV by landscape

Detection of AIV was associated with landscape (peri-urban vs. rural) when calculating prevalence at the stall level. We found that the prevalence of AIV in peri-urban regions (54.35%; 95% CI: 43.63–64.78) was significantly higher than in rural regions ($p < 0.01$). Additionally, we observed a significantly higher detection of subtype A/H5 (16.30%; 95% CI: 9.42–25.46) in peri-urban landscapes, at the stall level ($p = 0.03$; Figure 3).

3.2.3. Prevalence of AIV subtype by chicken strain

In our study, we sampled two types of chicken: Broiler and Sonali. On average, Sonali chickens were more positive for all subtypes compared to broiler chickens. Likewise, overall prevalence of AIV was significantly higher in the Sonali strain (55.26%; 95% CI: 43.41–66.69) with a value of p less than 0.01. HA/untyped was also more significantly associated with the Sonali strain (p = 0.03), (32.89%; 95% CI: 22.54–44.63; Figure 4).

3.2.4. Prevalence of AIV subtypes in fecal and offal samples

In total, we collected 139 pooled fecal swabs throughout our study. Of these samples, 50 tested AIV positive (35.97%; 95% CI:

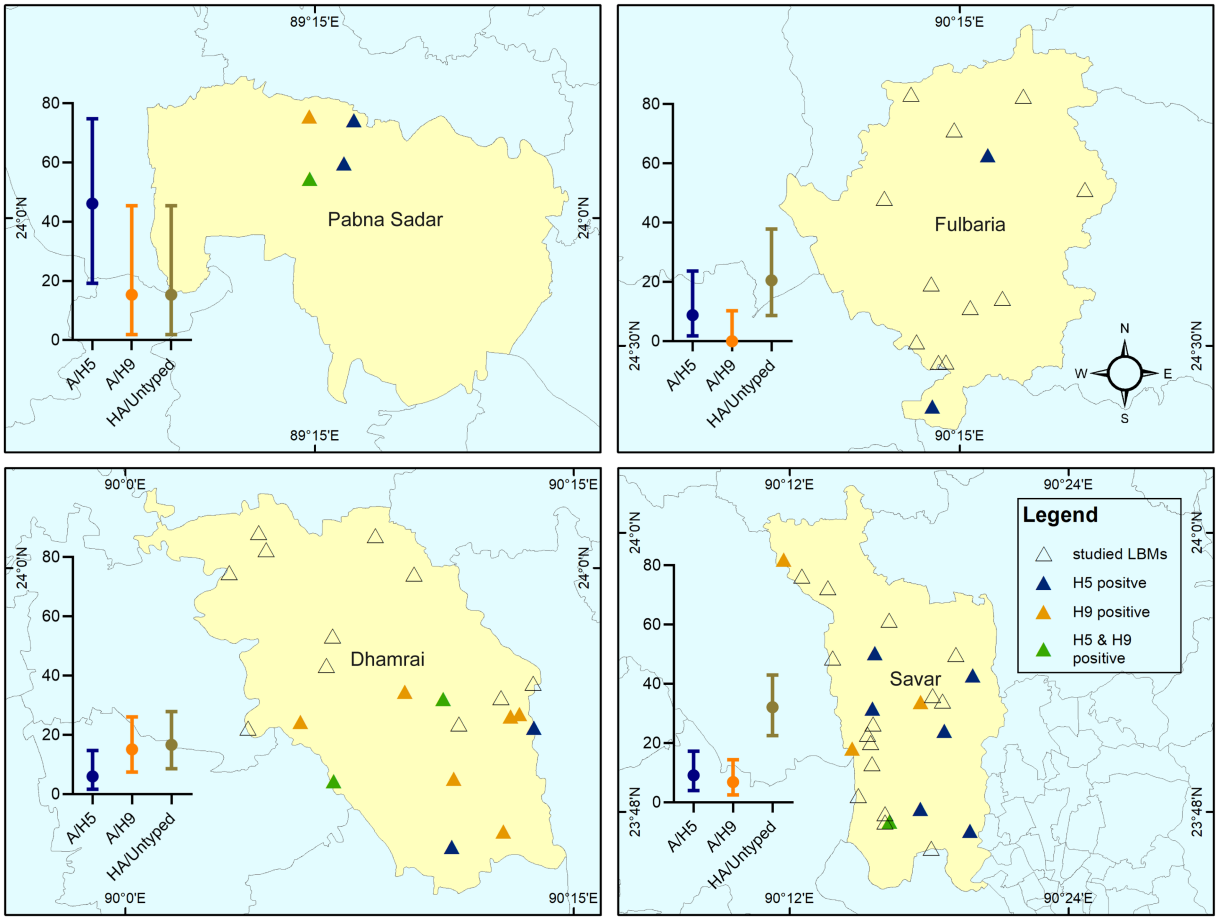

FIGURE 2
Spatial distribution of studied LBMs and the prevalence of H5, H9, and A/HA untyped detected in the LBMs. The error bar with mean value indicates the prevalence of subtypes of AIV at stall level in that region.

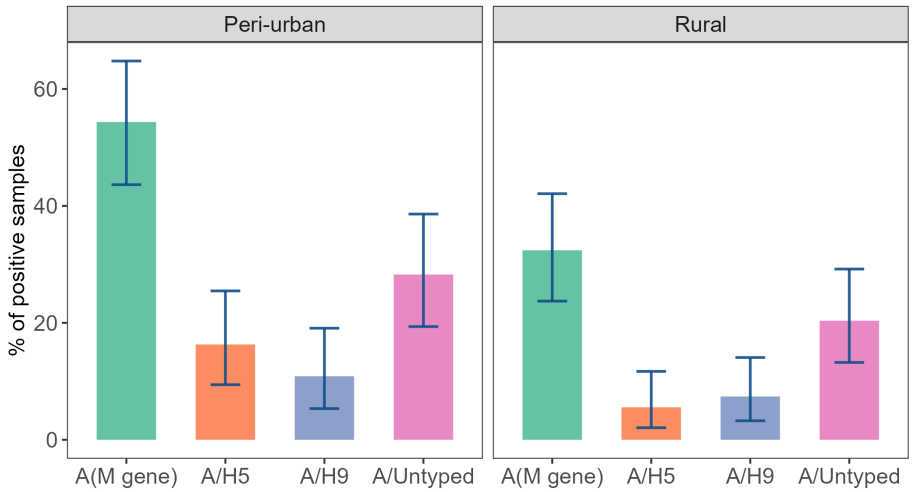

FIGURE 3
Prevalence of AIV subtypes in the stall across peri-urban and rural landscapes of studied LBMs.

28.01–44.54). We also collected a total of 61 offal swabs, 35 of which tested AIV positive (57.38%; 95% CI: 44.06–69.96) ($p < 0.01$). Detection of A/H5 and A/H9 was more common in offal samples (57.38%) than fecal (Figure 5). Additionally, the prevalence of A/untyped was significantly higher (36.07; 95% CI: 24.16–49.37) in the offal sample ($p = 0.01$; Figure 5).

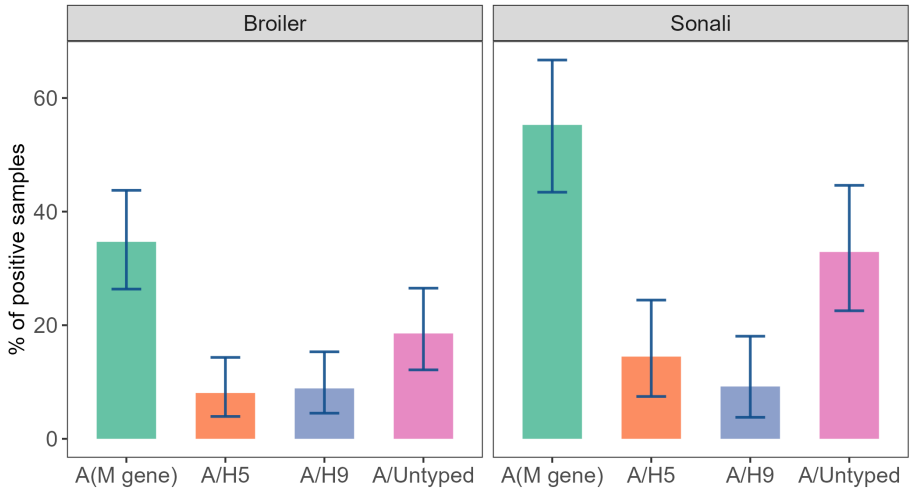

FIGURE 4
Diversity and prevalence of AIV subtypes in Sonali and Broiler in studied LBMs.

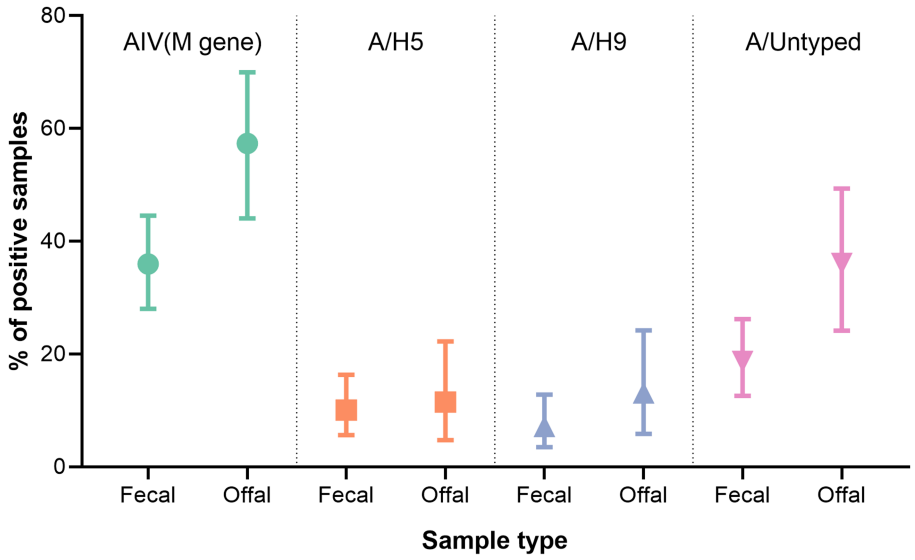

FIGURE 5
Diversity and prevalence of AIV subtypes in collected sample types in the study area.

3.3. Stall-level association between biosecurity practices and AIV circulation

3.3.1. Factors associated with AIV circulation using univariable analysis (results from Pearson's chi-square test)

We considered 21 stall-level variables related to hygiene and sanitation practices that could be associated with AIV contamination, circulation, and persistence in LBMs. We considered a stall as AIV positive if any of the samples collected from the stall tested positive for AIV or any of the subtypes. We then extracted 21 variables related to biosecurity from the questionnaire to analyze for association with AIV positivity at the stall-level. We found that 13 variables were significantly associated with detection of AIV at a 5% significance level in the univariate analysis. The landscape (rural vs. peri-urban) was significantly associated with AIV prevalence (Figure 3; Table 2). Stalls with a single chicken breed had a lower prevalence (28.74%; $p < 0.01$) compared to stall with more than one breed. Likewise, stalls that sold ducks and chickens were more positively associated with AIV (68.60%; $p = 0.03$). We detected a significant difference in prevalence of AIV for the stalls that kept birds on the floor compared to a cage (48.09%; $p = 0.04$), as well as for stalls that used bamboo boundaries compared to brick (49.09%; $p = 0.05$). AIV prevalence was also significantly higher among stalls in which the vendor answered yes to purchasing their birds from a middleman (57.66%; $p < 0.01$), and to disposing of their waste or dead birds in an open place (57.01%; $p < 0.01$). Vendors who used water instead of detergent as a cleaning agent had a significantly higher prevalence (54.41%;

$p < 0.01$). Stalls with unsold birds that remained overnight had a significantly higher prevalence (55.29%; $p = 0.01$) of AIV. Vendors that did not separate their sick birds from their healthy birds (59.62%; $p < 0.01$), did not prevent dogs from accessing the stall (55.88; $p < 0.01$), or wild birds from accessing the stall (58.33%; $p < 0.01$) had a higher prevalence (Table 2).

3.3.2. Matrix of Cramer's V to check for multicollinearity

Cramer's V measures the strength of an association between two variables. The coefficient ranges from 0 to 1. Where 0 means no association and 1 means perfect association. A value greater than 0.5 is considered a strong correlation between two variables (45). Figure 6 represents the matrix of values for Cramer's V between the explanatory variables. There were no pairs of variables with a Cramer's V value above our cut off point (0.5).

3.3.3. Multivariable modelling using a generalized linear mixed model

We conducted a GLMM with the variables found to be significant in the univariable analysis (Table 3). We included market as a random effect in our mixed-effect model since stalls were clustered by LBM. Poultry stalls in the peri-urban LBMs were at 3.02 times (95% CI: 1.18–7.72, $p = 0.02$) higher odds of AIV detection than the rural LBMs. The odds of AIV detection were 2.40 times higher for stalls with multiple chicken breeds (95% CI: 1.01–5.67, $p = 0.05$) compared to stalls with only one breed. The source of the birds was also found to be asscociated with AIV detection, in our model, with a middleman source at 2.35 higher odds compared to commercial farms (95% CI: 1.0–5.53, $p = 0.05$). Stalls where vendors did not separate their sick birds were at 3.04 times (95% CI: 1.34–6.92, $p = 0.01$) higher risk of infecting with AIV. Vendors who discard their waste and dead birds in open places rather than in dustbins had stall with 3.16 times (95% CI: 1.41–7.05, $p < 0.01$) higher risk of AIV contamination at the LBM surface. Most notably, vendors who did not use disinfectant to clean stall surfaces had 5.99 times (95% CI: 2.26–15.82, $p < 0.01$) higher odds of AIV detection. Lastly, we found 2.52 times (95% CI: 1.12–5.70, $p = 0.03$) higher risk of AIV where dogs had access to stalls and 2.31 times (95% CI: 1.01–5.30, $p = 0.05$) higher risk where vendors observed wild bird around their stalls (Table 3).

4. Discussion

AIV is a public health concern in the countries like Bangladesh, where people and poultry are in frequent contact without adequate biosecurity measures in place (32). LBMs are a significant source of AIV circulation, mutation, and spillover to humans or other wildlife. Over the past years, several studies have demonstrated a rising trend in AIV circulation among LBMs in Bangladesh (4, 46). However, these studies have almost exclusively targeted urban settings (7, 14, 22, 30). When we conducted our study from 2017 to 2018, only 36.63% of the total population of Bangladesh lived in urban areas, with the majority residing in peri-urban and rural areas (47). Furthermore, meat production

TABLE 2 Univariable analysis of factors to check association with AIV circulation (results from Pearson's chi-square test).

	Total	Prevalence (%)	95% CI	*p* value
Land type				
Peri-urban	92	50 (54.35)	43.63–64.78	<0.01
Rural	108	35 (32.41)	23.72–42.09	
Number of chicken strain keeping				
Multiple	113	60 (53.1)	43.48–62.55	<0.01
Single	87	25 (28.74)	19.54–39.43	
Flooring system				
Concrete	90	37 (41.11)	30.84–51.98	0.83
Mud	110	48 (43.64)	34.2–53.42	
Birds location at the stall				
Cage	69	22 (31.88)	21.17–44.21	0.04
Floor	131	63 (48.09)	39.28–56.99	
Boundary made of				
Bamboo	110	54 (49.09)	39.43–58.8	0.05
Brick	90	31 (34.44)	24.74–45.2	
Source of birds				
Farm	89	21 (23.6)	15.24–33.78	<0.01
Middleman	111	64 (57.66)	47.92–66.98	
Remain unsold overnight at the stall				
No	115	39 (33.91)	25.35–43.33	0.01
Yes	85	46 (54.12)	42.96–64.98	
Separate sick birds				
No	104	62 (59.62)	49.54–69.13	<0.01
Yes	96	23 (23.96)	15.83–33.75	
The bird died in the last seven days at the stall				
No	141	50 (35.46)	27.59–43.95	<0.01
Yes	59	35 (59.32)	45.75–71.93	
Disposal of offal and dead birds				
Burry/ Dustbin	93	24 (25.81)	17.29–35.92	<0.01
Throw away	107	61 (57.01)	47.08–66.54	
Cleaning agent				
Detergent	64	11 (17.19)	8.9–28.68	<0.01
Water only	136	74 (54.41)	45.66–62.97	
Running water supply at LBM				
No	139	57 (41.01)	32.74–49.66	0.63
Yes	61	28 (45.9)	33.06–59.15	
Are the worker/owner drink the same water				
No	172	71 (41.28)	33.84–49.02	0.51
Yes	28	14 (50)	30.65–69.35	
Dedicated cloth to work in the stall				

(Continued)

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TABLE 2 (Continued)

	Total	Prevalence (%)	95% CI	*p* value
No	111	49 (44.14)	34.73–53.88	0.70
Yes	89	36 (40.45)	30.17–51.38	
Use of mask				
No	180	78 (43.33)	35.98–50.91	0.63
Yes	20	7 (35)	15.39–59.22	
Drainage system				
No	137	55 (40.15)	31.87–48.86	0.40
Yes	63	30 (47.62)	34.88–60.59	
Electricity at stall				
No	79	28 (35.44)	25–47.01	0.14
Yes	121	57 (47.11)	37.97–56.39	
Closing day of the stall				
No	110	55 (50)	40.32–59.68	0.03
Yes	90	30 (33.33)	23.74–44.05	
Access to rodents at the stall				
No	98	35 (35.71)	26.29–46.03	0.08
Yes	102	50 (49.02)	38.99–59.11	
Access to a street dog				
No	98	28 (28.57)	19.9–38.58	<0.01
Yes	102	57 (55.88)	45.71–65.71	
Observe wild birds (nuisance birds) around the stall				
No	104	29 (27.88)	19.54–37.53	<0.01
Yes	96	56 (58.33)	47.82–68.32	

p-value <0.05; statistically significant.

and consumption has drastically increased in response to the growing density of the population over the years (48). As a result, there are now many more LBMs and commercial farms in rural and peri-urban areas. To address this critical gap in the research, we set out to investigate risk factors of AIV in peri-urban and rural LBMs in Bangladesh.

At the market-level, the overall prevalence of AIV was 82.54% (95%CI: 70.90–90.95). This is higher than the values reported by Sayeed et al. (31) in the Chittagong Metropolitan Area, where they detected an overall prevalence of 40% (95% CI: 20–60%; $N = 40$). This value is also higher than the measures of AIV prevalence reported in LBM studies outside of Bangladesh, in China and Indonesia specifically (49). Findings from this study indicate a higher prevalence of AIV in LBMs in peri-urban and rural areas in Bangladesh compared to previous studies conducted in urban areas of Bangladesh as well as in other Asian countries.

We detected AIV subtypes A/H5, A/H9, and HA/Untyped in at leasat one of the LBMs included within our study. We also found evidence of co-infection with A/H5 and A/H9 at the LBM level. Similar findings have been reported by other studies conducted at urban LMBs in Bangladesh (7, 30, 50). At the stall-level, 40.5% of the stalls included in our study tested positive for AIV, which is higher than in previous research conducted in Dhaka (24%), Chattogram (20.3%), and in a country-wide estimate (26%) (6, 30, 31). Our detected prevalence is also higher than in other countries in Asia (51–53).

4.1. Physiographic and hygienic status of poultry stalls in the LBMs

Our findings provide detailed biosecurity and hygienic practices at peri-urban and rural LBMs, which have previously been unexplored for AIV. We found that most vendors mixed multiple breeds of chicken in their stalls, and nearly 1/5 of vendors used the same cage

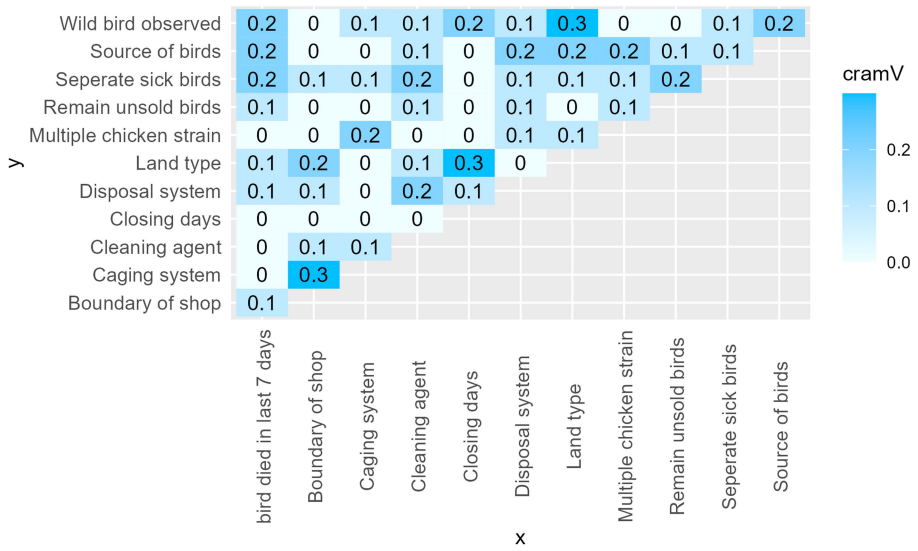

FIGURE 6
Cramer's V values matrix between explanatory variables.

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TABLE 3 Stall level generalized linear mixed model (GLMM) model of bio-security practices and AIV circulation in peri-urban and rural LBM.[a]

	Adjusted odds ratio (95% CI)	*p* value
Land type		
Rural	Reference	
Peri-urban	3.02 (1.18–7.72)	0.02
Number of chicken strains sold		
Single	Reference	
Multiple	2.4 (1.01–5.67)	0.05
Bird location in the stall		
Cage	Reference	
Floor	1.95 (0.79–4.77)	0.15
Boundary made of		
Bamboo	Reference	
Brick	0.62 (0.26–1.52)	0.3
Source of birds		
Farm	Reference	
Middleman	2.35 (1–5.53)	0.05
Remain overnight at stall		
No	Reference	
Yes	1.36 (0.61–3.04)	0.45
Separate sick birds		
Yes	Reference	
No	3.04 (1.34–6.92)	0.01
The bird died in the last seven days at the stall		
No	Reference	
Yes	1.3 (0.52–3.24)	0.58
Disposal of offal and dead birds		
Burry/Dustbin	Reference	
Throw away	3.16 (1.41–7.05)	<0.01
Cleaning agent		
Detergent	Reference	
Water only	5.99 (2.26–15.82)	<0.01
Weekly closing day of the stall		
No	Reference	
Yes	0.66 (0.29–1.5)	0.32
Dog access at the stall		
No	Reference	
Yes	2.52 (1.12–5.7)	0.03
Observe wild birds (nuisance birds) around the stall		
No	Reference	
Yes	2.31 (1.01–5.3)	0.05

[a]Stall level multivariable generalized linear mixed model was adjusted for cluster effect (LBM).

for chickens and ducks. Birds were frequently kept on the floor, and remained overnight if unsold. Many vendors did not separate their sick birds from their healthy birds or dispose of their biological waste properly, often discarding in open places. A limited number of vendors used detergent to clean their stall surfaces or utensils. They did not have dedicated clothes for their daily activities, and in some cases, there was no drainage system at all. Rodents and dogs can freely enter the stalls in most markets, and vendors noted the recurring presence of wild birds. Studies conducted by Chowdhury, Azziz-Baumgartner (30) and Sayeed, Smallwood (31) noted similar conditions among urban LBMs in Bangladesh.

4.2. Risk factors associated with the circulation of AIV in the stall of the peri-urban and rural areas

The odds of AIV infection in poultry are three times higher in peri-urban LBMs compared to rural LBMs in our study. These odds are twice as high as those found in a similar study conducted in Vietnam, which concluded that peri-urban areas had a 1.5 times higher risk of AIV than rural areas (54). With the population density in peri-urban areas, they need more nutrition, and one of the primary sources is poultry. To meet the growing demand, vendors collect their birds from inter-district or middlemen, where rural LBMs can cover the demand from nearby or backyard farms (52). Moreover, higher poultry density implies higher risk, and proximity to the highway increases the possibility of trading poultry from distant areas, which could increase the spread of AIV in peri-urban settings (54, 55).

Selling more than one breed of poultry within the stall was found to be significantly associated with AIV infection, which is aligned with the findings of Chowdhury et al. (30). Keeping multiple poultry species provides a suitable environment for effectively transmitting and amplifying AIV and allows it to spread over a large geographic area (50). In our study, birds kept on the floor rather than in cages were at greater risk of AIV. A study in Pakistan also found that keeping birds outside cages was a risk factor (56). When birds move openly on the floor, they keep in touch with the same surface that could be infected by the other sick birds (57). Additionally, birds on the floor are more likely to interact with terrestrial wild birds (58). The virus is less likely to spread in cages by keeping the birds isolated. Also, the layer of caging restricts feces and utensils from getting everywhere (59).

The source of birds is also considered a risk factor for AIV. Most vendors in our study collect their birds from a middleman, so there is no information on the conditions of transport or storage prior to purchase. As a result, birds purchased *via* middlemen could have been exposed to conditions which are conducive to virus transmission (51). A study in Vietnam showed that trading live birds from different sources is associated with reduced biosecurity and consequently, higher viral transmission (60). Keeping sick birds in contact with healthy ones was found to be a risk factor for AIV in our study. This is consistent with findings from a similar study in Uganda (61). The Food and Agriculture Organization (FAO) of the United Nations recommends separating sick and healthy birds as a requirement for biosecurity in LBMs (62). In peri-urban and rural areas, vendors usually throw away biological waste in the nearby pit or drain around the stall and market (63). Improper waste disposal management can facilitate opportunites for environmental

exposure to AIV (64). If offal and dead birds are discarded in open areas, rodents and other birds (e.g., crows) can easily reach them, causing a significant risk for healthy birds (61).

Of all the risk factors examined in our study, the cleaning agent was found to be the most strongly associated with AIV positivity. If vendors used only water, compared to detergent/disinfectant, they had much higher odds of exposure to AIV. Detergent is essential to inactive the virus, whereas water cannot disinfect the surface properly (65). Detergent has been demonstrated to effectively inactivate AIV from wood, tiles, and hard surfaces (66). Conversely, previous studies have showed that water could not adequately eliminate AIV contamination (67). If stalls are easily accessible to dogs and wild birds, they may provide an ideal environment for virus spillover. Stray dogs and wild birds frequently eat offal or dead birds that might be infected with AIV, and they could travel to other stalls or LBMs, which threatens market biosecurity (68, 69). Several studies found unusual crow die-off events in Bangladesh due to AIV, and LBMs were considered a primary infection source (10, 12, 61). In contrast to urban areas, dogs and wild birds have easy access to stalls because most markets have no boundaries. As a result, dogs and wild birds can be carriers of AIV, and we should take steps to prevent stray dogs and wild birds from entering LBMs.

5. Conclusion

Our study demonstrates a high prevalence of AIV, with evidence of subtype H5 and H9 circulating in peri-urban and rural LBMs in Bangladesh. We identified several unhygienic practices and factors associated with detection of AIV that should be considered for future interventions or educational materials in LBMs. Most stall owners are unaware of the risks associated with mixing species, choice of caging, inadequate waste disposal, and improper disinfection. Viral transmission often goes unnoticed in LBM settings in peri-urban and rural areas, so many vendors lack a tangible motive to improve biosafety protocols. Based on the findings presented here, LBMs demonstrate inadequate safety measures to prevent viral transmission, particularly in peri-urban and rural settings. We recommend continuous awareness building and monitoring of hygienic practices at LBMs in the peri-urban and rural areas to prevent the spread of AIV to poultry, people, and wildlife in Bangladesh.

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 study protocol was approved by the Institutional Ethics Committee of the Institute of Epidemiology Disease Control and Research (IEDCR/IRB/2015/04). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Chattogram Veterinary and Animal Sciences University-Animal Experimentation Ethics Committee (protocol: CVASU/Dir (R&E) AEEC/2015/751).

Author contributions

AI and JHE: conceptualization. AI, SI, and MKR: methodology. AI and MI: software, formal analysis, visualization, and writing—original draft preparation. AI, SI, MI, and MMH: validation. AI, MKR, and MAS: investigation. MZR, AI, and MS: resources. AI, MKR, SI, and MI: data curation. AI, SI, MKR, SM, MAS, TS, and MMH: writing—review and editing. MZR, MEH, and MAS: laboratory support. TS, MSF and JHE: supervision. MSF, TS, JHE, and MZR: project administration 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

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Emerging and re-emerging zoonotic viral diseases in Southeast Asia: One Health challenge

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The ongoing significant social, environmental, and economic changes in Southeast Asia (SEA) make the region highly vulnerable to the emergence and re-emergence of zoonotic viral diseases. In the last century, SEA has faced major viral outbreaks with great health and economic impact, including Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), arboviruses, highly pathogenic avian influenza (H5N1), and Severe Acute Respiratory Syndrome (SARS-CoV); and so far, imported cases of Middle East Respiratory Syndrome Coronavirus (MERS-CoV). Given the recent challenging experiences in addressing emerging zoonotic diseases, it is necessary to redouble efforts to effectively implement the "One Health" initiative in the region, which aims to strengthen the human-animal–plant-environment interface to better prevent, detect and respond to health threats while promoting sustainable development. This review provides an overview of important emerging and re-emerging zoonotic viral diseases in SEA, with emphasis on the main drivers behind their emergency, the epidemiological situation from January 2000 to October 2022, and the importance of One Health to promote improved intervention strategies.

KEYWORDS

Southeast Asia, zoonoses, emerging and re-emerging viral diseases, drivers, One Health, epidemiology

1. Introduction

Emerging and re-emerging infectious diseases are defined as recently recognized or evolved, or previously identified that have shown significant changes in their geographic, host or vector range (1). More than 60% of the emerging infectious diseases are zoonoses originating from domestic animals, poultry, livestock, and increasingly (71.8%) from wildlife species (2).

Zoonoses are caused by various pathogens, such as bacteria, viruses, parasites or prions that are naturally transmitted from vertebrate animals to humans during spillover events. In particular, viral infections pose a major threat to human health, as they can be transmitted by

aerosol, direct contact with animals or their fluids, through food or vectors (3), and it is estimated that more than 1.6 million unknown viral species of mammalian and waterfowl can infect humans, of which up to half have zoonotic potential (4).

Zoonotic transmission involves the interaction of a pathogen and at least two host species: (a) a natural reservoir, infected with the pathogen and often asymptomatic (shedding the pathogen), (b) a recipient host, presenting the disease (infected with the pathogen from a different host), and (c) an intermediate host, that may or may not be present, acting as a bridge or mixing vessel (vertebrate or invertebrate vector). Pathogens can be transmitted to the recipient host (humans) directly from the natural reservoir, from the intermediate vertebrate or invertebrate host, or from the environment, resulting in transmission to humans without spread ("dead-end spillover"), or in adaptation for human-to-human transmission (5). Although these events are relatively rare, in the last century, outbreaks of emerging and re-emerging viral zoonoses have increased in frequency and magnitude with significant human and animal health impacts, as well as incalculable and far-reaching economic consequences, as a result of the intensification of the animal-human interface, driven primarily by anthropogenic factors (6).

Their unpredictable emergence, their potential to cause severe diseases in humans and animals, and the frequent absence of effective vaccines and antiviral treatments, make their containment difficult. Therefore, our ability to predict and prevent future outbreaks depends on recognizing, understanding, and mitigating this complex and multifactorial process, which involves the interaction of animals, environment, pathogens, and humans, creating a favorable environment for interspecies transmission. However, to effectively achieve these actions, collaboration and transdisciplinary partnerships are required.

The World Health Organization (WHO), World Organization for Animal Health (OIE), and Food and Agriculture Organization (FAO), belonging to the Tripartite collaboration, have been working together for years, and in 2022 became quadripartite with the support of the United Nations Environment Program (UNEP) (7), to mainstream "One Health," defined by WHO as "an approach to designing and implementing programs, policies, legislation and research in which multiple sectors communicate and work together to achieve better public health outcomes" (8). This approach supports countries to improve prevention, monitoring, detection, control and containment of zoonotic diseases while contributing to sustainable development (7) (Figure 1 illustrates the spillover events of selected zoonotic viral diseases from the natural reservoir to humans, influenced by drivers that promote their emergence and re-emergence, and the "One Health" initiative).

Southeast Asia (SEA) is a sub-region of Asia, within the tropical climatic zone, comprising the Association of Southeast Asian Nations (ASEAN) (Brunei, Singapore, Malaysia, Thailand, the Philippines, Indonesia, Vietnam, Lao People's Democratic Republic (PDR), Cambodia, and Myanmar), and one observer state (Timor-Leste) (9). The region is politically, culturally, and socioeconomically diverse, undergoing major environmental, economic, and social changes (10), which have triggered a number of emerging and re-emerging zoonotic viral diseases during the last century (Table 1). The region is increasingly embracing "One Health." However, there are still significant barriers that vary from country to country and hinder its successful implementation.

The present review provides an update on emerging and re-emerging zoonotic virus diseases in SEA on (1) the drivers of their emergence, (2) the epidemiology of Severe Acute Respiratory Syndrome (SARS-CoV), Highly Pathogenic Avian Influenza (HPAI) H5N1, Middle East Respiratory Syndrome Coronavirus (MERS-CoV), Chikungunya virus (CHIKV), Zika virus (ZIKV), and Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) between January 2000 and October 2022, (3) The success story of HPAI H5N1 in Thailand, and (4) lessons learned from previous diseases, and One Health.

2. Drivers of zoonotic viral diseases in Southeast Asia

SEA is a hotspot for zoonotic diseases caused by changes in the modern human population dynamics that disrupt the environment, such as population growth and travel, deforestation, agriculture and meat production, wildlife consumption and trade, as well as climate change (34).

2.1. Population growth and travel

The population of SEA has grown considerably, from 360 million in 1980 to more than 680 million inhabitants in 2022, representing 8.6% of the total world population. Social development has led to a decline in fertility, from 4.5 births/woman in 1980 to 2.1 in 2021, and to an improvement in mortality rates, with life expectancy raising from 60.4 years old (yo) in 1980 to 70.2 yo in 2021. However, gaps remain wide between countries (35).

The region has highly populated countries, such as Indonesia, the fourth most populous in the world, and densely populated countries such as Singapore, ranking third worldwide with 8,700 people per km^2 (35). Urbanization is another notable population trend, driven by access to higher education, job opportunities, and health security. Currently, half of the SEA population lives in urban areas, and it is expected to exceed 70% by 2050 (36).

Migration has also emerged as a significant factor influencing population dynamics. Approximately 9.2 million migrants of working age live in Singapore (37% of the total population), Brunei (25.5%), Malaysia (15.0%, including undocumented migrants), and Thailand (5.2%, including undocumented migrants), with 77.2% of them coming from other ASEAN countries (37).

Population growth is closely related to emerging and re-emerging viral zoonotic diseases in many ways, such as high human density allows diseases to spread faster; the number of human births, immunologically naïve individuals, increases the risk of re-emerging diseases or depletion of immunity when vaccines are available; and old adult populations may increase viral transmission due to the lower capacity of the immune system to contain diseases. On the other hand, other human requirements also increase the risk, including housing (urbanization of untouched ecosystems), which comes along with overcrowding and low-quality dwellings, and increased migration of people (6, 38).

Tourism is a major source of income in SEA, which has grown from 63 million visitors in 2009 to 139 million in 2019 (before COVID-19 emergency), according to the United Nations World Tourism Organization (UNWTO) (39, 40), with almost 40 million

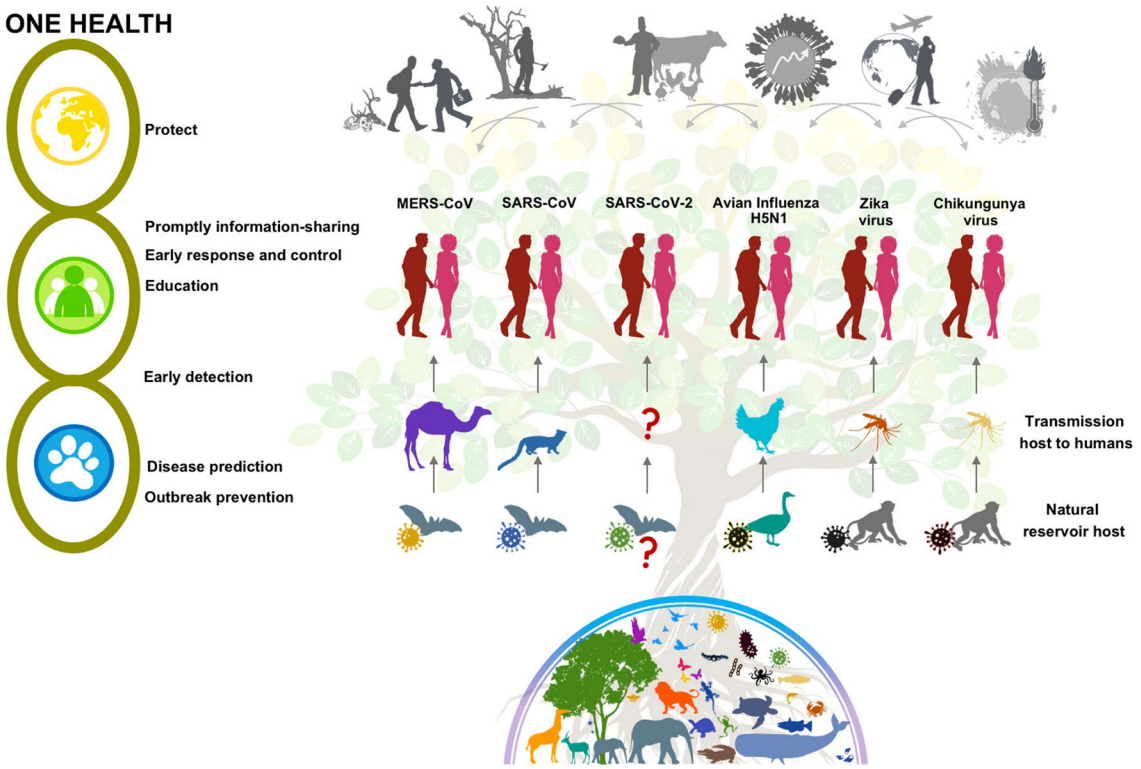

FIGURE 1
Emerging and re-emerging zoonotic viral diseases. Drivers, reservoir hosts, transmission to humans, and One Health action. Wildlife trade and consumption, deforestation, agriculture and meat production, population growth and urbanization, global travel, and climate change are well documented drivers that have contributed to the emergence and re-emergence of zoonotic viral diseases. Zoonotic spillover events are a complex mechanism that requires the interaction of a natural animal reservoir infected by a pathogen and often asymptomatic, a recipient host, which presents the disease, and a transmitting or intermediate host (vertebrate or invertebrate vector) that may or may not be present, acting as a bridge or serving as a mixing vessel. In the last century, several zoonotic viral diseases have emerged or re-emerged including: Middle East Respiratory Syndrome (MERS-CoV), Severe Acute Respiratory Syndrome (SARS-CoV) and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2): Like SARS and MERS, SARS-CoV-2 has been hypothesized to have evolved from a strain found in bats. The main intermediate animal host responsible for human infection of SARS-CoV is the palm civet, for MERS-CoV the dromedary camel, and for SARS-CoV-2 it is still unknown. Highly pathogenic avian influenza (HPAI) H5N1: Wild waterfowl are the natural reservoir of low pathogenic avian influenza (LPAI) viruses, when transmitted to terrestrial poultry, LPAIVs can mutate into HPAI and be transmitted to humans. Zika virus (ZIKV): Transmission includes the sylvatic cycle, including non-human primates and arboreal canopy-dwelling *Aedes* mosquito species, and the urban cycle, including humans and mainly *A. aegypti* (the most competent) and *A. albopictus* mosquitoes. Chikungunya virus (CHIKV): Transmission includes the sylvatic cycle among forest-dwelling *Aedes* spp. mosquitoes and mainly non-human primates in Africa; and the urban cycle, maintained by mosquitoes (*A. aegypti* and *A. albopictus*) carrying the virus from human to human. One Health is a collaborative, multisectoral, and transdisciplinary approach that reinforces the human-animal–plant-environment interface to better prevent, predict, detect, and respond to health threats.

visitors in Thailand (5th in the world), 26 million in Malaysia, and 19 million in Singapore in 2019, generating 147.6 billion U.S. dollars in tourism receipts in 2019 (41, 42).

This good connectivity allows the spread of diseases to other continents in short periods, the introduction of new vectors into suitable environments, or new pathogens into vector populations, amplifying the risk of new outbreaks or pandemics (39, 40).

2.2. Deforestation

SEA has around 15% of the world's tropical forests, and at least four of the 25 biodiversity landscapes (43). Unfortunately, deforestation is a pressing problem throughout the region, where at least 1.2% of its forests are lost annually (44) driven mainly by farming (73%), and logging (19%), with the fear that more than 40% of total forest area could disappear by 2,100 (43, 45). For example, a large loss in tree cover has been observed from 2001 to 2021, where Indonesia has lost 28.6 million hectares (Mha) (18% decrease in tree cover since 2000), Malaysia 8.67Mha (29%), Myanmar 4.3Mha (10%), Lao PDR 4.05Mha (21%), and Cambodia 2.60Mha (30%) (46).

The most important cause of deforestation in SEA is palm oil production, which has increased enormously, accounting for 90% of global production (45% of plantations were forests in 1989). In particular, Indonesia is the main producer (followed by Malaysia), contributing to the country's economy and providing job opportunities to 4 million people (45, 47, 48); consequently, it is the worst-affected country in the region, in addition to having experienced a massive fire in 2016 that accounted for 30% of all tree cover loss for that year and was reportedly deliberately started by small-scale farmers to clear lands. Cambodia, on the other hand, recently records the highest percentage of total forest loss worldwide due to poor forest management (between 2001 and 2010 88,000 ha/year, and 2011–2021: 155,000 ha/year) (46, 49, 50).

TABLE 1 Emerging and re-emerging zoonotic viruses in Southeast Asia in the XXI century (January 2000–October 2022).

Virus	Year of first human case* Year of major outbreak** (per country)	Country	Reference
SARS-CoV	2003	Vietnam	(11)
		Singapore	
		Thailand	
		The Philippines	
		Indonesia	
		Malaysia	
Influenza A(H5N1)	2003	Vietnam	(12)
Influenza A(H5N1)	2004	Thailand	(12)
Influenza A(H5N1)	2005	Indonesia	(12)
		Cambodia	
Dengue virus	2007	Cambodia	(13)
Influenza A(H5N1)	2007	Lao PDR	(12)
		Myanmar	
Chikungunya virus	2008	Malaysia	(14)
Chikungunya virus	2009	Thailand	(15)
Ebola	2009	The Philippines	(16)
Influenza A(H1N1)	2009	All SEA countries	(16)
Chikungunya virus	2009	Myanmar	(17)
		Indonesia	(18)
Dengue virus	2013	Thailand	(15)
		Lao PDR	(19)
Chikungunya virus	2013	Singapore	(21)
		Lao PDR	(20)
Dengue virus	2014	Brunei	(22)
MERS-CoV	2014	Malaysia	(23)
MERS-CoV	2015	Thailand	(23)
		The Philippines	
Dengue virus	2015	Myanmar	(24)
Dengue virus	2016	Indonesia	(25)
Zika virus	2016	Singapore	(26)
		Thailand	(15)
		Vietnam	(105)
Chikungunya virus	2017	The Philippines	(27)
Dengue virus	2019	Malaysia	(29)
		The Philippines	(28)
Monkeypox	2019	Singapore	(16)
SARS-CoV-2	2020	All SEA countries	(30)
Chikungunya virus	2020	Cambodia	(31)
Dengue virus	2020	Singapore	(32)
Monkeypox	2022	Vietnam	(33)
		Thailand	
		The Philippines	
		Indonesia	
Dengue virus	2022	Vietnam	(32)
		Timor-Leste	

SARS-CoV, Severe Acute Respiratory Syndrome; MERS-CoV, Middle East Respiratory Syndrome; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; PDR, People's Democratic Republic; SEA, Southeast Asia.
*Year of first human case: SARS-CoV, Influenza A(H5N1), Ebola, Influenza A(H1N1), MERS-CoV, Monkeypox, SARS-CoV-2.
**Year of major outbreak: Zika virus, dengue virus and Chikungunya virus.

Among the many consequences of deforestation and forest degradation, including loss of biodiversity [SEA among the most threatened regions, at least 221 terrestrial and freshwater vertebrates are critically endangered (51)], climate change [12–20% of global greenhouse gas emissions (GHG)], and loss of soil fertility, are zoonotic diseases, which particularly affect countries in the intertropical zone with high forest cover. It causes environmental stress on wildlife, an impact on the reservoir host (e.g., survival of generalist and opportunistic species along with their pathogens) and/or vector populations dynamics that favor transmission, as well as increased animal-human interaction (52–54).

2.3. Agriculture expansion, meat production, wildlife consumption and trade

Agriculture in SEA is an important source of economy and livelihood, except for Singapore and Brunei, contributing to more than 10% of the gross domestic product (GDP), and employs one-third of all workers (55). The region is among the main producers of rice, vegetable oil, and sugar (56). Rice production is the main crop and accounts for 26% of global production and 40% of world exports, with Cambodia, Indonesia, Myanmar, the Philippines, Thailand and Vietnam being the main producers per crop (57). Maize is the second most produced cereal, which is also the primary source of feed for the poultry and livestock industry, and has the largest harvested area in Indonesia, the Philippines, Thailand, and Vietnam (58). Furthermore, Thailand has become the world's leading exporter of rubber (56, 59).

Meat production has increased enormously; in particular, between 2009 and 2018 poultry farming has expanded by 56%, and pig farming has increased by 23%, especially in Vietnam and Thailand. Moreover, Indonesia is the largest egg producer (56, 59). At the same time, wild animals are hunted indiscriminately and removed from their natural habitat through legal and illegal trade. They are traded for collectibles, food (for some it represents status and prestige, believed to be "healthier" or simply for their "wild taste") and served in restaurants

in Vietnam and Cambodia, but also as sale items, pets, medicinal, in open-air wet markets, or through online platforms and social media (53, 60). To illustrate, more than 3,000 parts and products of the critically endangered Helmeted Hornbill were seized, especially in Indonesia between 2010 and 2019, over 96,000 kg of pangolin scales in Malaysia, Singapore and Vietnam between 2017 and 2019, and 45,000 live birds in Indonesia in 2018–2019 (51).

Agriculture is responsible for GHG emissions, loss of biodiversity, deforestation, increased water demand, increased release of reactive nitrogen into the environment, and allows pathogens to jump species by enabling the movement of animals, the exchange of products and services, the confinement of animals in close contact and stressful conditions, and through the consumption of wild meat (61).

2.4. Climate change

The region is one of the most vulnerable to climate change, facing warming trends and a possible alteration of the South Asian monsoon pattern. These changes are primarily attributed to the use of fossil fuels, deforestation, and agricultural practices (62). Annual temperatures have increased by about 0.6°C per decade over the past 100 years (63), and weather events have increased in number and intensity. According to the 2022 world risk index report, the Philippines (1st worldwide), Indonesia (3rd), Myanmar (6th), and Vietnam (12th) had the highest estimated disaster risk (64), while according to the long-term climate risk index between 2000 and 2019, Myanmar was the most affected country in SEA and the second in the world, where Cyclone Nargis in 2008 was the worst natural disaster ever recorded in the country, and at least the second deadliest globally, responsible for 140,000 deaths and catastrophic destructions, followed by the Philippines which is recurrently affected by tropical cyclones such as Pablo (Bopha) in 2012, Yolanda (Haiyan) in 2013, Ompong (Mangkhut) in 2018, and Odette (Rai) in 2021 (65).

The increase in global temperature or the length of the seasons also affect the geographic distribution and density of species. Particularly, it influences the transmission dynamics of vector-borne infections, increasing the survival, reproduction, and abundance of vector populations (6).

3. Epidemiology of emerging and re-emerging viral zoonoses in Southeast Asia (January 2000–October 2022)

3.1. Severe acute respiratory syndrome coronavirus

In November 2002, SARS-CoV first emerged in Guangdong Province, China. Bats have been recognized as the natural reservoir, and the palm civet, as the intermediate animal host (Figure 1). Early cases were detected in patients who lived near a market or were food handlers (66, 67). The epidemic spread within Guangdong with a high rate of transmission among health care workers (HCWs), before spreading to Hong Kong in February 2003, through an infected HCW who stayed in a hotel and caused infection in at least 16 guests and visitors. The movement of infected people, caused other outbreaks within and outside the country (68). The SARS pandemic ended in July 2003, and caused over 8,000 infections and 774 deaths in 29 countries, with a case fatality rate (CFR) of 9.5%, and about 50.0% among patients aged >65 years. Five additional zoonotic cases were confirmed between December 2003 and January 2004 (11, 69).

Six countries in SEA reported a total of 331 human cases of SARS-CoV infection (4.1% of global cases) and 44 deaths (CFR = 13.3%) between late February and May 2003. Transmission was initiated by the hotel guests and caused mainly nosocomial infections in Singapore (total cases $n = 238$; imported = 8; CFR = 13.9%; HCWs = 41.0%) (68), in Vietnam (total $n = 63$; imported = 1; CFR = 7.9%; HCWs = 57.0%) (11, 68), and in the Philippines (total $n = 14$; imported = 7; CFR = 14.3%; HCWs = 28.6%), the latter caused by a nursing assistant, derived from an outbreak in Toronto, Canada (70). However, Thailand ($n = 9$, CFR = 22.2%), Malaysia ($n = 5$; CFR = 40.0%), and Indonesia ($n = 2$; CFR = 0.0%) reported only imported cases (11) (Figure 2A).

3.2. Middle East respiratory syndrome coronavirus

In June 2012, a patient with MERS was first reported in Jeddah, Saudi Arabia. However, a cluster of undiagnosed severe respiratory illness among HCWs in Jordan in April 2012 was later confirmed to be caused by MERS. Like SARS, MERS evolved from bats (71), and the dromedary camel acted as the intermediate animal host responsible for human infection (Figure 1) (72). The disease has spread within (e.g., Qatar) and outside the Middle East, including the Republic of Korea, where in 2015 a traveler caused a large outbreak in 16 healthcare settings (73, 74). As of 31 October 2022, 2,600 human cases of MERS have been reported (Saudi Arabia = 84.3% of cases), with 935 deaths (CFR = 36.0%) in 27 countries. Transmission occurred mainly among patients in healthcare settings (62–79%) (73, 75).

Between 2014 and 2017, seven imported human cases (0.3% of global cases) with one death (CFR = 14.3%) of MERS were confirmed in three SEA countries. Three cases in Thailand, the first and second were in older men from Oman in June 2015 and June 2016, who arrived for other medical reasons, and the third in July 2016 in a young Kuwaiti man who arrived for vacation (76), two cases in Malaysia, in men returning from a pilgrimage in Saudi Arabia, in April 2014 (the first death), and in December 2017 (77, 78), and two in the Philippines, in January 2015, in a Filipino nurse working in Saudi Arabia (79), and in July 2015 in a male from Finland who traveled to Saudi Arabia and United Arab Emirates before arriving in the country (80) (Figure 2B).

3.3. Chikungunya virus

Chikungunya virus (CHIKV) was first isolated in Tanzania in 1952 from the serum of a febrile patient. Since then and for the next 50 years, sporadic cases were reported in Africa and Asia. CHIKV has recently been transmitted globally on all continents except Antarctica, affecting millions of people each year, especially in all tropical and several subtropical areas (81, 82). Transmission includes both sylvatic and urban cycles. The sylvatic cycle among forest-dwelling *Aedes* spp.

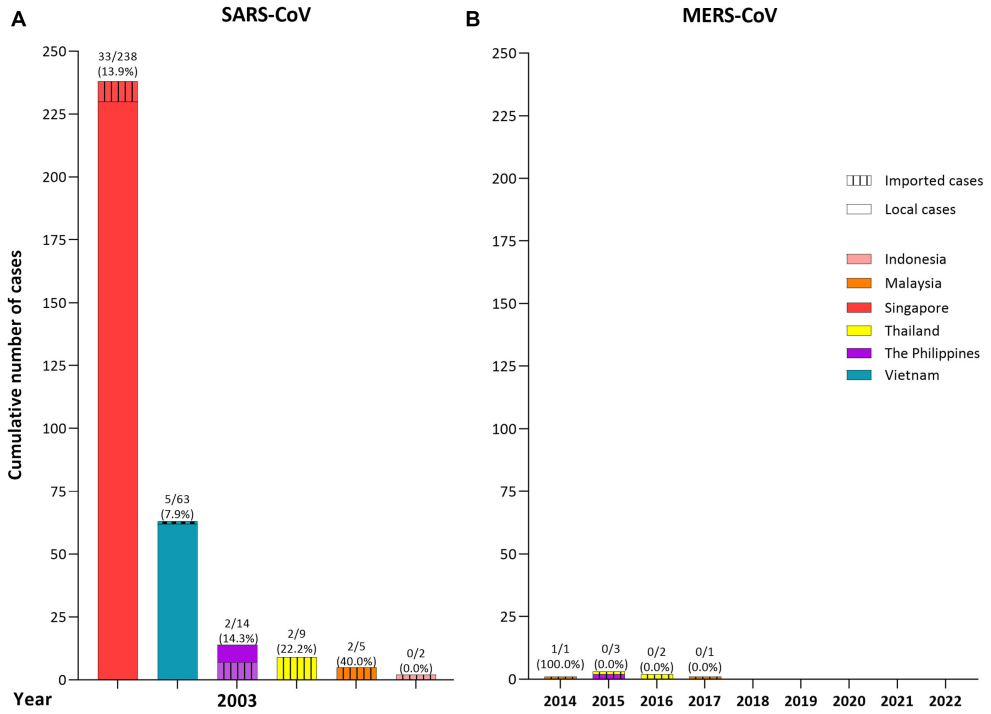

FIGURE 2
Cumulative number of cases (local or imported) of human Severe Acute Respiratory Syndrome (SARS), and Middle East Respiratory Syndrome Coronavirus (MERS) in Southeast Asia (SEA) per country and per year (until October 2022). **(A)** SARS: 331 human cases (4.1% of global cases) and 44 deaths were reported between February and May 2003 in 6 countries. Imported cases and local transmission were reported in Singapore, Vietnam, and the Philippines, while only imported cases were reported in Thailand, Malaysia, and Indonesia. **(B)** MERS: 7 imported cases (0.3% of global cases) with one death were reported between 2014 to 2017 in 3 countries, Thailand, Malaysia, and the Philippines. The number in bar charts represent: number of deaths/total number of cases per country (% of mortality).

mosquitoes and mainly non-human primates in Africa (including Guinea baboons, Chacma baboons, African green monkeys, patas monkeys, red-tail monkeys, guenons, bushbabies, and mandrills (83)), and the urban cycle, maintained by mosquitoes (*Aedes aegypti* and *Aedes albopictus*) carrying the virus from human to human, observed in the Americas, Asia, the Indian Ocean, and Europe (84, 85) (Figure 1).

In SEA, CHIKV emerged in Bangkok, Thailand in 1958 (although evidence suggests earlier transmission in Indonesia (86)), followed by other minor outbreaks or sporadic cases in other countries such as Cambodia (1961), the Philippines (1965), Vietnam (1966–1967), and Indonesia (official report 1972), with no major outbreaks between the 1980s and 2000s (81, 87). CHIKV re-emerged in the region in the 21st century. Some countries suffered large and multiple outbreaks, mainly after 2008, while others experienced low-level circulation. Indonesia has been the most affected country (25), followed by Thailand (15), Malaysia (14, 88), the Philippines (88–90), Cambodia (31, 91), and Singapore (92). While Myanmar (17, 93), and Lao PDR (20, 94) reported minor outbreaks or sporadic cases, and no available data are available for Vietnam, Brunei and Timor-Leste (the number of cases of the two largest outbreaks per country is reported in Figure 3). However, the true burden of CHIKV virus disease remains unknown. The number of cases is often underreported, due to the limited laboratory diagnosis, lack of accurate reports to health authorities, limited surveillance programs, and co-circulation with dengue virus on some occasions that may mask CHIKV infections (20, 95).

3.4. Zika virus

Zika virus (ZIKV) was isolated in 1947 from a sentinel rhesus monkey in the Zika forest of Uganda, and in humans in 1952 in Nigeria. The virus is maintained in two cycles: the sylvatic cycle, including non-human primates and arboreal canopy-dwelling *Aedes* mosquito species, and the urban cycle, including humans and mainly *A. aegypti* (the most competent) and *A. albopictus* mosquitoes (96) (Figure 1).

Serological evidence suggests that ZIKV circulated a low but at sustained levels in African countries from 1945 to 2014, and in Asian territories from 1952 to 1997 (97, 98). The first known outbreak occurred in 2007 in the Yap State of Micronesia, followed by the Pacific Islands in 2013–2014. Subsequently, major outbreaks occurred in Latin America and the Caribbean between 2015 and 2016. ZIKV has affected more than 87 countries and territories worldwide (99, 100).

ZIKV has been circulating in SEA since at least the 1950s based on neutralization assays. However the first human case was confirmed in 2010 in Cambodia (101). Epidemiological data are limited, with outbreaks reported between 2016 and 2018 in Thailand (2,300 cases) (102, 103), Singapore (458 cases) (26), and Vietnam (265 cases) (104, 105). Studies evidence low-level circulation in the Philippines, Myanmar, Lao PDR, Cambodia, Malaysia, and Indonesia (101, 102, 106–108), and no data are available for Brunei and Timor-Leste (Figure 3).

Overall, laboratory-confirmed and probable ZIKV cases do not estimate the total number of cases, which are often

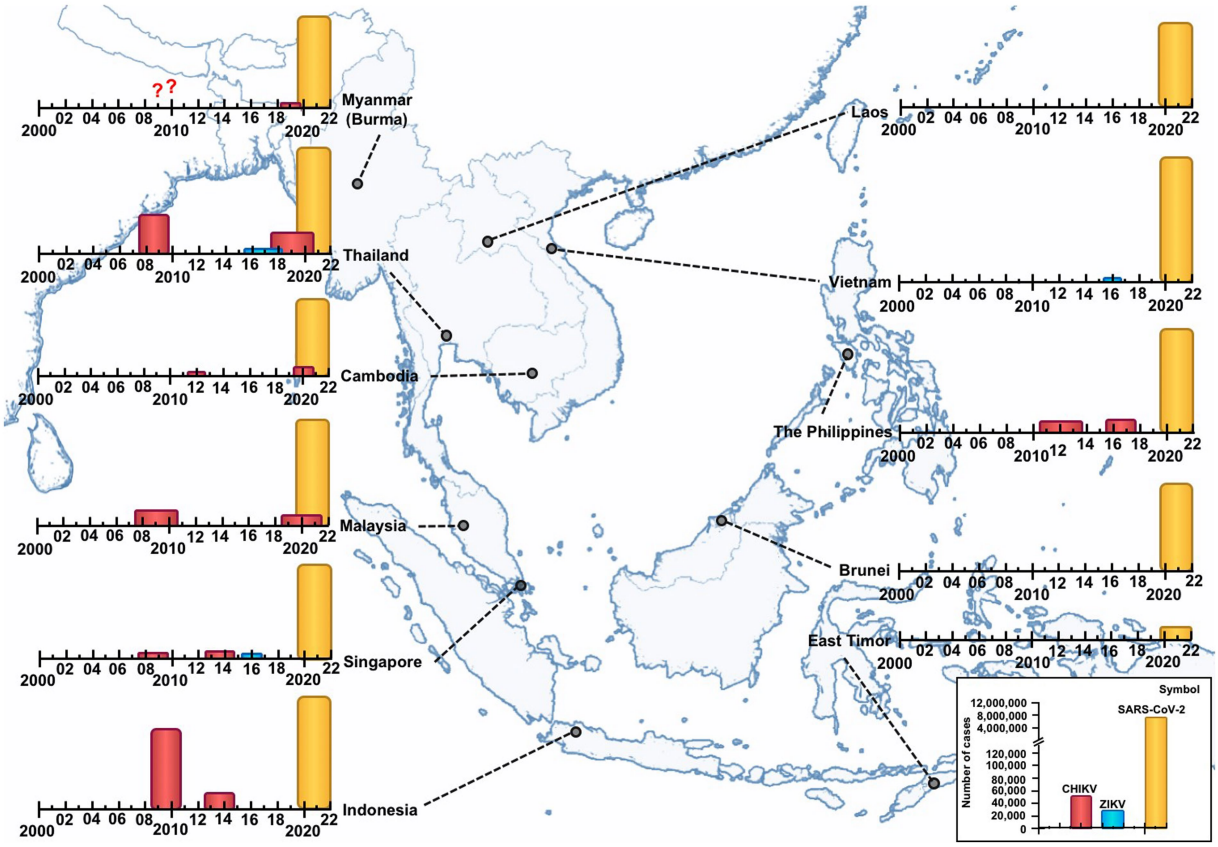

FIGURE 3
Epidemics of Chikungunya virus (CHIKV) (two major), Zika virus (ZIKV), and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in Southeast Asia (SEA) per country (January 2000–October 2022). CHIKV has caused several outbreaks of varying magnitude and scope. The cumulative number of cases of the two major recorded outbreaks per country is as follows: Indonesia: 2009–2010=135,000 cases and 2013–2014=~22,500 cases. Thailand: 2008–2009=54,000 cases and 2018–2020=17,000 cases. Malaysia: 2008–2010=10,500 cases and 2019–2021=~5,000 cases. The Philippines: 2011–2013=2,800 cases and 2016–2018=11,500 cases. Cambodia: 2011=190 cases and 2020=>6,000 suspected cases. Singapore: 2008–2009=1,059 and 2013–2014=1,241. Myanmar and Lao PDR=few cases. Vietnam, Brunei and East-Timor: no data. ZIKV outbreaks were limited and were reported between 2016 and 2018, in Thailand (2,300 cases), Singapore (458 cases), and Vietnam (265 cases). SARS-CoV-2: East-Timor, Cambodia and Lao People's Democratic Republic reported the lowest number of confirmed cases (23,305, 137,995 and 150,000 cases, respectively); in contrast, Indonesia and Vietnam reported the highest number, with 7 and 11 million, respectively. ??: no data of cases.

underestimated due to asymptomatic and pauci-symptomatic cases and combined with similar clinical presentation of other diseases such as dengue fever. In addition to misinterpretation of serological data as consequence of extensive cross-reactivity between flaviviruses that must be confirmed by seroneutralization tests (101).

3.5. Severe acute respiratory syndrome coronavirus 2

Recently, in December 2019, SARS-CoV-2 the etiologic agent of COVID-19 (Coronavirus disease 2019) emerged in Wuhan City, Hubei Province, China, with unidentified pneumonia cases associated with a wholesale seafood market. The virus is believed to have evolved from bats (71). However, for a bat viral pathogen to successfully emerge in humans, it usually requires an intermediate host, which remains unknown in the case of SARS-CoV-2 (Figure 1) (72). The virus spread rapidly to all continents, leading the WHO to declare a global pandemic in early March 2020 (67). To date, the pandemic has caused more than 629 million confirmed cases and 6.5 million deaths worldwide (109).

SEA was one of the first affected regions, with cases reported in January 2020 in Thailand, Vietnam, Malaysia, Cambodia, Singapore, and the Philippines (110). To date, more than 35 million confirmed cases (Figure 3) have been reported with a CFR of 0.7. Currently, Myanmar has the highest CFR (3.1%), while Brunei and Singapore have the lowest CFR (~0.1%) (30). Among cumulative cases per 100,000 inhabitants, Brunei and Singapore top the list. In contrast, Cambodia and Myanmar are at the bottom of the list (111). It should be noted that the number of cases is underestimated in several countries, due to underreporting of cases, related to poor testing, asymptomatic infections or mild symptoms (112).

Since December 2020, vaccines have played an important role in the COVID-19 pandemic. At this time, Brunei (99.9%) and Singapore (93.9%) have the highest number of fully vaccinated people, unlike Myanmar (51.2%) and Indonesia (62.4%), which have the lowest vaccination coverage (113).

3.6. Highly pathogenic avian influenza

H5N1 virus emerged in 1996 in farmed geese in Guangdong Province of southern China (People's Rep. of), as the goose/Guangdong lineage (Gs/Gd), followed by an outbreak in poultry in Hong Kong in 1997 that resulted in the culling of 1.3 million chickens. The virus evolved from low pathogenic avian influenza (LPAI) viruses present in wild waterfowl (natural reservoir). LPAIVs, when transmitted to terrestrial poultry, can mutate into highly pathogenic avian influenza (HPAI), and can "spillback" to wild birds, which can carry and shed the viruses (114) (Figure 1). Since its emergence, the H5N1 subtype has evolved and diversified into multiple phylogenetic lineages (clades), given the segmented nature of the influenza viral genome that confers evolutionary advantages (115, 116).

In 2003, H5N1 reemerged and caused several outbreaks in SEA, including Vietnam, Thailand, Cambodia, Laos, Indonesia, Malaysia, and Myanmar, mainly among backyard poultry (61), and other Asian countries such as South Korea, Japan, Hong Kong, and China (117). Outside of Asia, the virus has spread to the Middle East, Africa, Europe, North America (Canada and United States of America), and recently to Latin America (118). Studies suggest that poultry and migratory birds were involved in the introduction into Asia and Africa, and migratory flyways of wild birds to Europe and America (119, 120).

Human cases were first reported in Hong Kong in 1997. The infection of three individuals returning from Fujian, China and the death of two of them marked the re-emergence in early 2003 (121). To date, it has caused 868 human cases (Egypt, 41.5% of cases) and 456 deaths (>50% mortality, varying between countries) in 21 countries worldwide, affecting mainly children and younger adults (122, 123).

The epidemiological data of human H5N1 demonstrate the high percentage of cases described in SEA ($N = 412$), corresponding to 47.5% of global cases, and high mortality rates with almost 70.0%. Several countries were affected such Indonesia (200 cases), Vietnam (127 cases), Cambodia (56 cases), Thailand (25 cases), Lao PDR (3 cases), and Myanmar (1 case), mainly between 2004 and 2014 (12) (Figure 4). Most human cases had a history of close contact with poultry (e.g., backyard farming systems, live bird markets, consumption of diseased poultry). Although clusters of limited human-to-human transmission have occurred after prolonged exposure to a symptomatic infected person, sustained human-to-human transmission has not yet occurred (61).

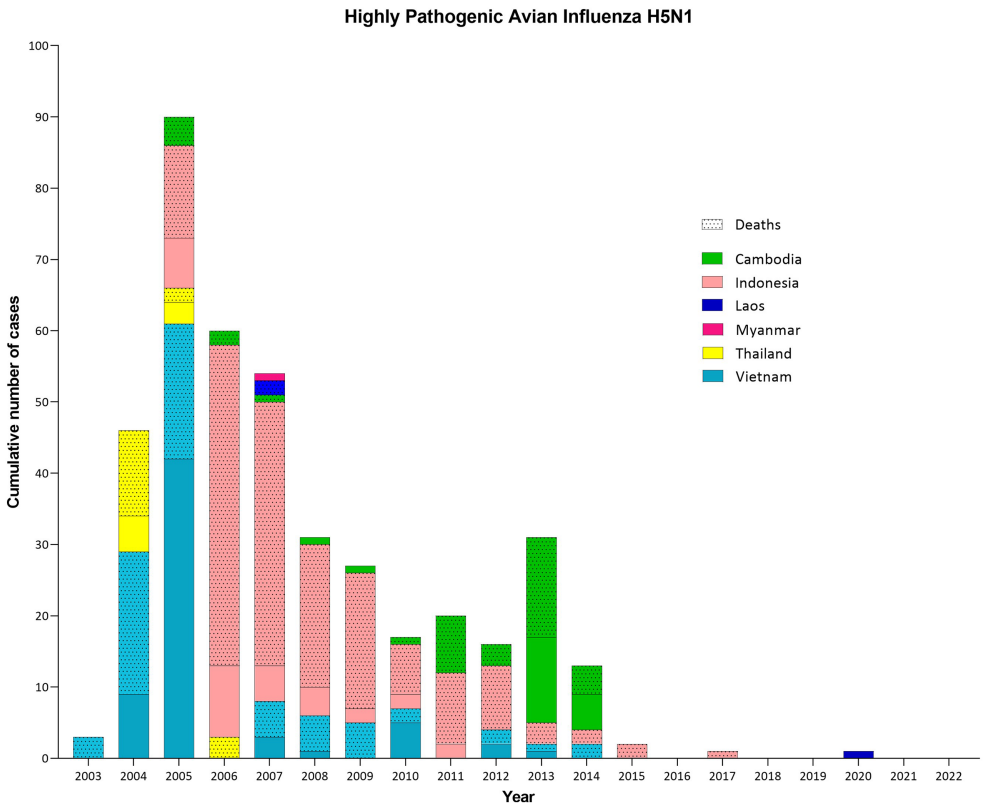

FIGURE 4
Cumulative number of confirmed human cases and deaths of Highly Pathogenic Avian Influenza H5N1 in Southeast Asia (SEA) per country and per year (until October 2022). Human cases of H5N1 in SEA represent almost half of the total number worldwide (412/868: 47.5%), with a high case fatality rate (69.9%). Between 2003 and 2020, cases were reported in 6/11 SEA countries, with 2005 being the most devastating year. Indonesia (200 cases and 168 deaths, CFR=84.0%), Vietnam (127 cases and 64 deaths, CFR=50.4%) and Cambodia (56 cases and 37 deaths, CFR=66.1%) have been the most affected countries, reporting intermittent cases until at least 2014, unlike Thailand (25 cases and 17 deaths, CFR=68.0%), which succeeded to report no human cases after 2006. In addition, Myanmar (1 case) and Lao PDR (3 cases and 2 deaths, CFR=66.7%) confirmed some cases.

4. The success story of highly pathogenic avian influenza in Thailand

Since 2003, the HPAI H5N1 virus has spread across SEA, causing unprecedented epidemics affecting poultry farmers, livelihoods, commercial poultry, tourism, and human health. In particular, Thailand has experienced several large epidemics during 2004 and 2005 in 60 of 76 provinces (124). However, the outbreaks were reduced to very low levels and no human cases were reported after 2006 due to effective control strategies (125).

4.1. Outbreak investigation

The virus was first confirmed on January 23, 2004 in Thailand, on a chicken farm in Suphanburi Province. This first wave lasted until May 2004, with 193 outbreaks in 42 of 76 provinces, affecting mainly chickens, but also broilers, layers, native chickens, ducks, geese, turkeys, ostriches, quail, and peacocks (126). The second large wave started on July 3, 2004, when H5N1 was confirmed on layer farms in Ayutthaya and Pathum Thani Provinces, and finished in March 2005 with a higher number of confirmed outbreaks (~1,492 outbreaks in 52 provinces), particularly in ducks and backyards chickens with poor hygiene. This higher number can be explained by the higher number of tests performed compared to the first wave (117). More than 62 million birds were either killed or culled for disease control (126). However, from July 2006 to 2011, there were few and limited outbreaks, mainly on small-scale backyard farms (124, 127).

In response, a program throughout Thailand was launched in mid-January 2004 to detect cases in sick or dead poultry (117). The Thai Department of Livestock Development (DLD) implemented several strategies to control epidemics including disease investigation, animal movement control, pre-emptive culling ("stamping-out"), disinfection, surveillance and quarantine (117, 126, 128), as described in Figure 5. The Thai government compensated farmers for their losses, which encouraged farmers to report outbreaks. In addition, improved detection, better hygiene, and better response after the first wave contributed to the decrease in the number of outbreaks in poultry (129, 130). Surveillance results during the first week identified that the virus was already circulating in many types of poultry throughout the north and the south (130), stating that the delay in the identification was too long and contributed to the large scale of the outbreaks.

Thailand prohibited vaccination, although black-market vaccines may have been used (131). Unlike other countries like Vietnam that used inactivated vaccines to stop outbreaks, but after a year without outbreaks, the country faced a major outbreak in 2007 and sporadic outbreaks since then. This situation confirms the difficulties in

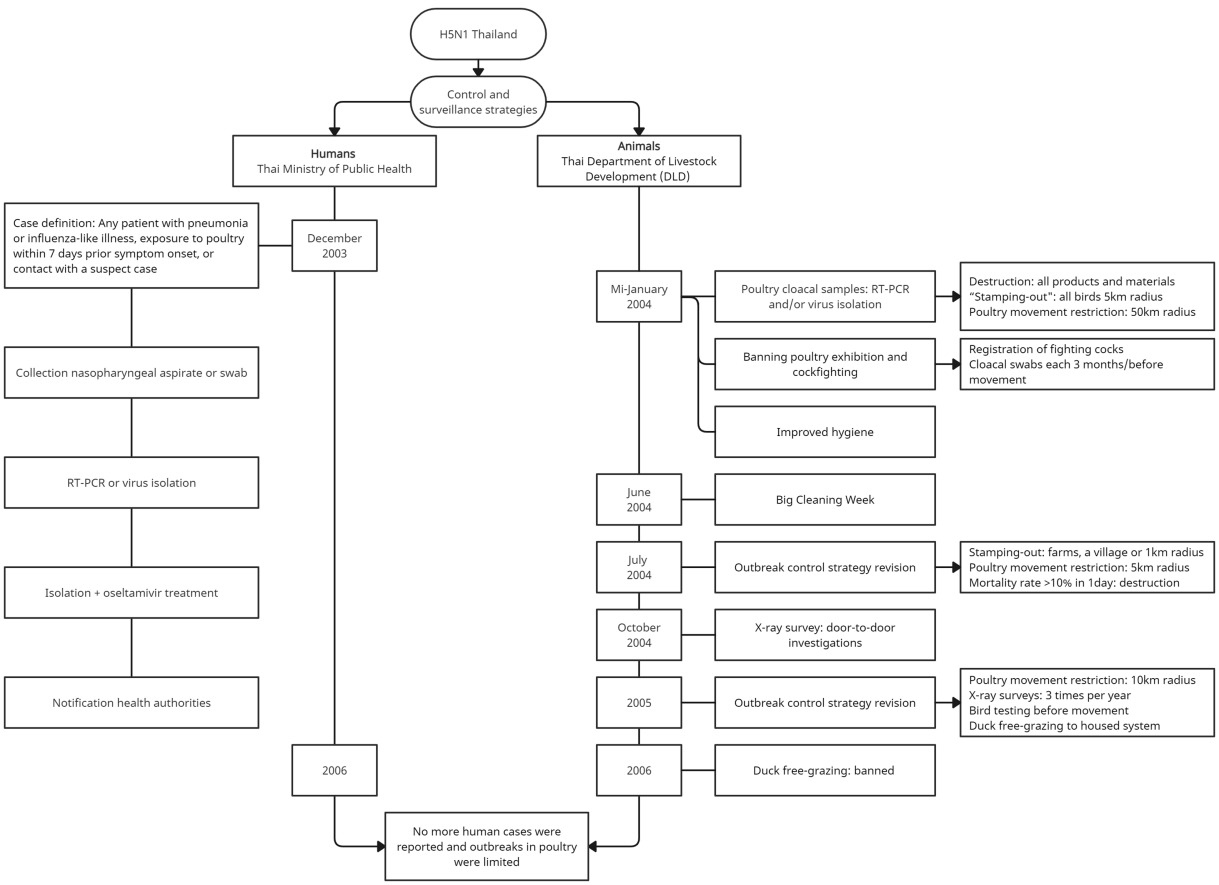

FIGURE 5
Highly pathogenic avian influenza H5N1 control and surveillance strategies in Thailand. The control and surveillance strategies implemented in Thailand during the large outbreaks in 2004–2005, limited the number of poultry outbreaks and human cases after 2006.

maintaining good flock immunity in poultry populations (132). Studies have shown that the H5 subtype is less immunogenic (133). However, vaccination can be effective for outbreak control when accompanied by strict monitoring and testing (117).

The Ministry of Public Health confirmed the first human cases in children with severe progressive pneumonia from Suphanburi and Kanchanaburi Provinces on the same day of detection in poultry, through the national surveillance program to investigate human cases (134, 135) (Figure 5). Human outbreaks occurred mainly in Central Thailand, affecting children, between January–March and August–October 2004. In September 2004, a probable person-to-person transmission was identified in a family cluster (135).

4.2. Tracing the origin of avian influenza H5N1

Virus introduction into Thailand could have been *via* migratory birds (119); its expansion through the transport of poultry and poultry products, and the trade in wild birds; and its persistence caused by free-grazing ducks and rice cultivation (130). In Thailand, the high density of poultry populations (commercial and backyard poultry sectors with low biosecurity), live bird markets, bird migration from central and northern Asia to Thailand, and festivals related to poultry production and movement, played an important role in the spread of the initial large-scale outbreaks, and made disease control difficult in 2004 (117, 128, 136).

4.3. Breaking the transmission chain by using a One Health program

Thailand has recognized the need to establish a surveillance system since 2005, implementing strategies, such as the National Strategic Plan for Avian Influenza Control and Pandemic Influenza Preparedness in Thailand 2005–2007, launched by the Thai Government. This plan was then succeeded by the Second National Strategic Plan for Prevention and Control of Avian Influenza and Preparedness for Influenza Pandemic BE 2551–2,553 (AD 2008–2010), and finally, Thailand adopted "One Health" for emerging infectious diseases into the national strategic plan (2013–2016) (137–139).

Thailand innovates with a specific government unit, the Coordinating Unit for One Health (CUOH), which is the coordinating center for One Health activities. Several collaborators, such as government agencies, academic institutions, and the private sector, work together to maintain communication and promote activities (e.g., surveillance, control, conferences, and training) (137–139). For instance, in 2016, Thailand piloted a One Health avian influenza surveillance system, which demonstrated strengths but also encountered challenges (140).

5. Lessons from previous emerging and re-emerging viral zoonoses, and One Health

Since the early 2000s, zoonotic diseases have caused considerable economic and human impact, and have highlighted the importance of surveillance, prevention and control, as well as the importance of coordination between the human and the animal health sectors. However, the COVID-19 pandemic has exposed long-existing gaps in addressing emerging diseases, including: (a) preparedness and prompt response, (b) public health infrastructure, (c) effective and rapid risk communication, (d) research, and (e) political commitment, and national and international collaborations (141), and recognized the interdependence of humans, animals, plants and environment that reinforced the relevance of One Health (142).

One Health is a solution for sustainable and equitable future by protecting and restoring ecosystems, preserving human and animal health, and providing long-term economic benefits. This initiative requires mobilization, communication, coordination and collaboration across multiple sectors, disciplines, communities, and all levels of government (143).

SEA is committed to One Health, but despite the substantial progress made in recent years, it remains challenging to address illegal wildlife trade, corruption, insufficient political regulations, funding, population growth (e.g., culture and education), illegal logging, climate change, the preservation of forests, ecosystems and species, deforestation (economic pressure generated by the agricultural sector that limits policies), infrastructure and data sharing (in the most vulnerable regions) (143).

To better implement this approach, it is necessary to establish or strengthen cross-sectional and transdisciplinary working groups to share knowledge, challenges, needs and solutions, and thus develop effective actions and plans. Learn about existing One Health tools, resources and frameworks to improve public awareness, including rural and indigenous communities (e.g., create incentives). Identify One Health funding opportunities to receive support for sustainable projects. Promote information sharing and training among SEA countries, including communication, leadership and health diplomacy. Improve existing agreements by encouraging investment in sustainable strategies (143, 144).

Therefore, preventing the next zoonotic pandemic requires a critical shift toward a sustainable, cost-effective and integrated approach.

6. Concluding remarks

Zoonotic diseases do not respect national borders and can rapidly spread across regions and countries. Southeast Asia is a hotspot for the emergence and re-emergence of zoonotic viral diseases induced mainly by land use changes resulting from agriculture and population growth, which threaten biodiversity, forests, and climate. Over the last century, the region has experienced zoonotic viral outbreaks of varying magnitude and scope; in particular, apart from the SARS-CoV-2 pandemic, large epidemics of Chikungunya virus, highly pathogenic avian influenza H5N1, and SARS have been reported, causing serious concern and enormous health and economic impact.

To reduce the spread of viral zoonotic diseases in SEA, increasing multidisciplinary networks between countries is important. However, several factors including different cultures and traditions, surveillance and control systems disparities between SEA countries can limit the sharing of information and resources. Therefore, it is important to address these challenges and implement effective prevention measures tailored to the region's specific needs and circumstances. The level of "One Health" education programs should increase, it may also involve educating the population about the

causes and transmission of diseases and promoting healthy behaviors and practices that can help to prevent the spread of viral zoonotic diseases.

Author contributions

PMSV, NG, and SW contributed to the conception, design of the study, and wrote the first draft of the manuscript. PMSV, NG, TS, SY, AM, PL, DM, and SW reviewed and edited the manuscript. All authors contributed to the manuscript revision, read, 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.

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The effect of antibiotic usage on resistance in humans and food-producing animals: a longitudinal, One Health analysis using European data

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This paper estimates the effect of antibiotic usage in humans and food-producing animals on the prevalence of resistance in zoonotic bacteria in both humans and animals. Using comprehensive longitudinal data from annual surveillance reports on resistance and usage in Europe, we find that antibiotic usage in food-producing animals and antibiotic usage in humans are independently and causally related to the prevalence of resistance in both humans and animals. The study considers simultaneous and total usage of antibiotics in humans and food-producing animals to identify the marginal effects and joint effects of usage on resistance of both groups. By employing lagged-dependent variable and fixed-effects specifications, we provide a lower and an upper bound on the effects on resistance. The paper also contributes to the scant literature on how antibiotic use in humans is related to resistance in other animals.

KEYWORDS

antibiotics, resistance, Europe, causality, interspecies effect, One Health

1. Introduction

Antimicrobial resistance not only poses a growing public health threat to humans but also risks animal health and production (1–3). Antimicrobial agents used in human medicine often belong to the same classes as those used in food-producing animals and many antimicrobials are used in both humans and animals (4, 5). Given the overlap of antibiotics used in these populations, there is a growing concern that the extensive usage of antibiotics in one population could contribute to the development of resistance to antibiotics commonly used in the other (6). The extent of this well-documented biological relationship is, however, not well characterized at an ecological level, i.e., across time and space, and a consensus is yet to be reached on the empirical connection between usage and resistance within and across humans and food-producing animals. It is crucial to approach this relationship from a One Health perspective since policies, regulations and stewardship in one sector can affect other interrelated sectors.

While usage of antimicrobials is a primary contributor to resistance, evidence suggests there are socioeconomic, institutional and environmental factors which also play a role (7–11). The issue also lies at the intersection of various scientific, technical, behavioral, ecological, and economic disciplines which renders forming informed approaches to alleviate the problem even more complex. Researchers from various fields are studying to gain a better understanding of

antimicrobial resistance but the diverse perspectives and innate complexities involved results in a lack of consensus and at times conflicting findings (12).

This paper therefore undertakes an empirical analysis of the relationships between use of antibiotics in human and animal populations and antibiotic resistance in both populations using national surveillance data. We evaluate four possible effects: use in animals causing resistance in animals; use in animals causing resistance in humans; use in humans causing resistance in humans; and use in humans causing resistance in animals, as indicated in Figure 1. The existing evidence on these effects is extensive, but also incomplete. The most critical issues relate to cross-species effects on resistance. For example, bovine respiratory diseases lead to heavy consumption of antibiotics, which may lead to increased prevalence of resistant infections in both livestock and humans (13). While detailed studies show clearly that farmers and their direct contacts working with livestock acquire antibiotic resistance genes that seem clearly related to the use of antibiotics in these animals, what is less obvious is whether there is a wider spread in the human population (6). Our study provides evidence on this relationship. Moreover, there exists mixed evidence about the sharing of resistance genes across humans, livestock, and the environment (14, 15). Therefore, direct sharing of resistant infections is not the only way that usage of antibiotics in one population can affect others.

1.1. Animal–animal

The extensive use of antibiotics in livestock contributes to the emergence of antibiotic-resistant bacteria in animal populations (2, 16, 17). Numerous studies have investigated the link between antibiotic usage in food-producing animals and antibiotic resistance in bacteria present in those animals. Studies primarily focus on national surveillance reports from European countries for a variety of combinations of pathogens, antimicrobial substances, and animal species (2, 16–18). The evidence from these studies suggests that there is a positive correlation between the amount of antibiotics used and the development of resistance in bacteria present in food-producing animals. Studies also show that reducing antibiotic usage in food-producing animals could lead to reductions in resistance in those animals (19–23).

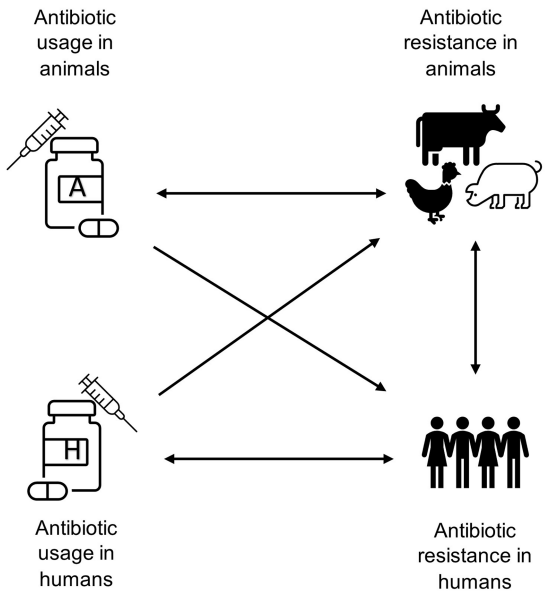

FIGURE 1
Schematic diagram highlighting the potential associations between antibiotic usage and antibiotic resistance in food-producing animals and humans.

1.2. Animal–human

While it is believed that the widespread use of antibiotics in food-producing animals is a significant source of antibiotic resistance in humans, the specific impact on human health is poorly understood (6, 24, 25). Resistant bacteria could be transmitted to humans through the consumption of undercooked or raw food, cross-contamination with other foods, or indirectly through the environment (26). Ceftiofur use in chickens was tied to resistant infections in humans in the province of Quebec, Canada, suggesting that transmission occurred through handling of raw meat (27). Direct transmission from animals to farm workers is also a concern (28–30). Alternatively, antibiotics intended for animals may be excreted and their presence in the environment may increase human exposure to resistant bacteria (26, 31, 32). The relationship between antibiotic usage in animals and antibiotic resistance in humans has been studied in the literature mainly by focusing on transmission pathways and using molecular analysis (14, 15, 24). There exists evidence of transmission pathways of resistant bacteria from animals to humans, but the quantitative and ecological extent of the problem is not yet fully understood (33). Few studies have examined the direct impact of antibiotic use in animals on the occurrence of resistant bacteria in humans. The extent of the effect on humans outside of the farm is still poorly quantified (25).

1.3. Human–human

There have also been numerous studies of the effect of antibiotic use in humans on resistance in humans. Several studies have focused on specific populations, such as a nursing home or hospital, and demonstrated that increased antibiotic use tends to precede increases in resistance locally (34–37). At an ecological level, the studies are mostly cross-sectional, and therefore offer limited opportunities for inferring causality (7, 11, 38–41). Our recent research uses longitudinal data to show that increases in antibiotic use nationally are followed by persistent increases in resistance for at least 4 years (42).

1.4. Human–animal

To our knowledge, there is only one other study that examines the relationship between use of antibiotics by humans and resistance in animals (43). However, other evidence has suggested that resistance in the environment may be affected by human medicines. For example, recent evidence suggests that use of oseltamivir in humans may result in environmental exposure for birds that in turn develop oseltamivir-resistant avian influenza virus (44).

An important omission in these studies is the recognition that antibiotic use in humans and animals is occurring simultaneously. In this situation, it is reasonable to consider usage of antibiotics in both animals and humans can potentially affect resistance levels in all species at the same time. Use in animals, for example, may affect humans *directly* through excretion of antibiotics into the environment; but it may also affect humans *indirectly* through the increase in antibiotic resistance in animals who then interact with humans, sharing antibiotic resistance genes. And the resistance in humans could easily be transferred back to animals. Given these parallel mechanisms allowing resistance to spread, a true "One Health" approach requires a holistic approach which accounts for use in humans and in other animals.

This paper thus brings together comprehensive data on usage in humans and food-producing animals of 11 antibiotic classes and occurrence of resistance in three bacterial species common in humans and food-producing animals from European surveillance reports over 11 years in 31 countries. This allows us to make numerous contributions to the growing literature on antibiotic use and resistance.

First, although antibiotic usage occurs simultaneously in humans and other animals, existing studies have almost exclusively considered the effect of antibiotic use in humans *or* other animals. Our study introduces an analysis with usage in both humans and food-producing animals, allowing us to identify the marginal effect of usage in humans and animals separately. We are also able to estimate their joint effect. Adda attempted to do a similar analysis using data from US states (38). However, his study relied heavily on extrapolation and interpolation of data and lacked state-level data on antibiotic use in animals as well as resistance data from animal sources.[1] Allel et al. conducted a recent cross-sectional study that considers usage in both humans and food-producing animals (43). While their analysis includes many more countries than ours, their data is cross-sectional in nature only and therefore cannot be used to address causality. We thus see these analyses as highly complementary and mutually reinforcing.

Second, our longitudinal data allows us to estimate the effect of usage on resistance in a causal framework, rather than just estimating correlations. Correlations can be informative, but it is hard to know from a cross-sectional analysis whether higher usage causes higher resistance or higher resistance causes higher usage. We use a methodology pioneered in the economics literature that allows us to bound the causal effect from use to resistance, though not to estimate it precisely.

Third, we show the effect of antibiotic usage in humans on resistance in food-producing animals at an ecological level. To our knowledge, only one other previous study has attempted to show how antibiotic use in humans is related to resistance in other animals (43).

1 To be more precise, Adda lacked state-level data on usage of antibiotics in animals and therefore projected national data onto each state using the value of sales of different food-producing animals. He did not attempt to assess the impact of antibiotic usage on resistance in animals.

2. Data and methods

2.1. Data on antibiotic resistance

Resistance data for our analysis is drawn mainly from the European Union Summary Report, published by the European Food and Safety Authority (EFSA), on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals, and food, which is published annually. EFSA along with the European Centre for Disease Prevention and Control (ECDC) prepares the report on the occurrence of antimicrobial resistance in isolates from human cases and in isolates from animals and foodstuffs. Participating laboratories of both member and non-member states of the European Union (EU) report their data on antimicrobial resistance. ECDC's protocols for harmonized monitoring and reporting of resistance in humans and food-producing animals are followed, during the reporting process, to overcome challenges of comparing antimicrobial resistance data from various countries using different laboratory methods and different criteria for interpreting resistance (45).

The reports provide resistance data for humans to specific antibiotic molecules for two important zoonotic pathogens, *Salmonella*, and *Campylobacter*. For *Salmonella,* data on total number of isolates tested and number of resistant isolates are available for several selected serovars of importance. The reports also provide similar data for two most important *Campylobacter* species. These reports, however, did not include data on resistance to *Escherichia coli* in humans. For this bacterial species, we therefore used data from the ECDC Surveillance Atlas of Infectious Diseases (46). We aggregated these total number of tested isolates and number of resistant isolates from different species of these pathogens, that were tested against specific antibiotics, at the genus level for each year and country. Then, using the total number of tested isolates and number of resistant isolates for each bacteria-antibiotic combination, we calculated the percentage of resistant isolates for each year and country. Data on human isolates were not present in the annual reports for the years 2008 and 2018. Thus, our data on antibiotic resistance in humans, given by the percentage of resistant isolates, vary by country, bacteria, and antibiotic class over the years 2009–2017.

On the animal side, resistance data to specific antibiotics molecules for *Salmonella*, *Campylobacter* and *Escherichia coli* were used. These are present in the EFSA annual summary reports (45). For *Salmonella and Campylobacter* resistance data is available for selected important species. Moreover, the isolates from different species of these pathogens originated from multiple sources, including live fowl, cattle, pigs and meat from the same species. The data across all these different sources from different bacterial species were aggregated at the genus level for each year and country. The data for animals is available for the years 2008–2018, but during this reporting period the sampling from the sources is not consistent across years. We calculated the percentage of resistant isolates using the total number of isolates and number of resistant isolates for 3 bacteria genera tested against specific antibiotics, for each year and country. Data on antibiotic resistance for food-producing animals vary by country, bacteria, and antibiotic combination and is available from 2008 to 2018.

Epidemiological cut-off values and clinical breakpoints are used to interpret resistance in human isolates from minimum inhibitory

concentration (MIC) data. MIC refers to the lowest concentration of an antimicrobial agent that is required to inhibit the growth of a microorganism under standard laboratory conditions (47). This measure is used to determine the susceptibility of microorganisms to antibiotics applying different methods, such as disk diffusion and broth dilution. A particular specimen is defined as resistant if it crosses a certain threshold of the MIC ratio. The annual reports use thresholds defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for detection of resistance for bacteria-antibiotic combinations included in this study (48, 49).

2.2. Data on antibiotic usage

Antibiotic usage data for humans were extracted from the IQVIA MIDAS database. IQVIA reports the total volume of sales of antibiotic molecules used in human medicine based on national surveys. We included only the antibiotic molecules that were also present in resistance data. Antibiotic molecules were then aggregated by class (e.g., fluoroquinolones) and quantified in metric tonnes. Thus, human antibiotic usage in tonnes varies by country and class and is available annually for the period 2008–2018. We extracted data on antibiotic usage in food-producing animals from the ESVAC database. The ESVAC project collects sales data of veterinary antimicrobials in participating European countries. The data is in tonnes for antibiotic class for 27 member states of the EU and 4 non-member states from 2008 to 2018 (50). Usage data for animals is available for 10 antibiotic classes, and, for humans for the same 10 classes plus *Carbapenems*.

2.3. Complementary data

We complement our data with control variables that vary by year and country. The control variables included are Population Correction Units for animals drawn from the ESVAC database (50), human population, Gross Domestic Product, and health expenditure *per capita* drawn from the World Bank Databank (51, 52), and the Corruption Perception Index from Transparency International (53). The Population Correction Unit is a standardized average weight in kilograms of all animals at the time of antibiotic treatment multiplied by the number of animals based on national statistics (50).

2.4. Sample definition

Merging these sets of data gives us an 11-year panel of data on usage in 11 antibiotic classes and on measured resistance to each class in 3 bacterial species in humans and food-producing animals in 31 European countries. In the early years, not all countries report, and over time the number of reporting countries increases. In addition, not all countries report resistance data on every combination of bacteria and antibiotic class for each year. Moreover, usage data is also not available for every member and non-member states for all years. This makes it an unbalanced panel. It should be noted that resistance data varies by year, country, bacteria, and antibiotic class, whereas usage data varies only by year, country, and class.

3. Methodology

3.1. Summary statistics and plots

We begin by summarizing the raw data. We calculate total usage in tonnes in humans and animals by class of antibiotics and find the average across years; and we calculate average resistance as the percentage of samples meeting ESVAC's resistance threshold by bacterial species.

We then show this data with greater granularity by plotting the relationship between log transformed resistance and usage data, using binned scatter plots and lines of best fit. To generate a binned scatterplot, the x-axis variable is grouped into equal-sized bins, then the means of the variables along both horizontal and vertical dimensions are computed within each bin. These means are used to create a scatter plot. We use the "binsreg" package in R to automatically determine bin sizes and compute corresponding means. Lines of best fit are plotted using ordinary least squares method, which provides the best linear approximation to the conditional expectation function. The plots explore the relationship between resistance and usage for food-producing animals and humans separately. First, we pool the data for an overview of the relationships and then explore relations by year, bacteria and class. These associations are also illustrated for each country in Supplementary material. The literature has extensive evidence on such correlation measures. However, the evidence is predominantly drawn from cross-sectional studies without accounting for time and controlling for confounding factors. Moreover, the findings are only relevant and limited to the particular samples and environments studied (2, 18, 29).

3.2. Regression analysis

Our main dependent variable is the natural log of the prevalence of resistance, as given by the percentage of resistant isolates. We estimate models separately using resistance data for food-producing animals and humans. The natural log of antimicrobial usage in tonnes, in food-producing animals and humans are our two main explanatory variables for identifying the marginal resistance effects arising from simultaneous usage. In addition, we also use the sum of antibiotic usage in food-producing animals and humans as the main explanatory variable in an alternative model which estimates the effect of combined usage on resistance for animals and humans.

To isolate the causal effect of antibiotic usage on antibiotic resistance we employ fixed effects and lagged dependent variable models. The effects on resistance from food-producing animals and humans are isolated separately. The models are presented below:

Fixed-effect specification:

$$\ln\left(Resistance_{i,t,g}\right) = \alpha + \beta \ln\left(Animal\_Use_{i,t}\right) + \gamma \ln\left(Human\_Use_{i,t}\right) + \theta_i + \lambda_t + \boldsymbol{X_{i,t}} + \varepsilon_{i,t}$$

Lagged-dependent variable (LDV) specification:

TABLE 1 Antibiotic usage by class for food-producing animals and humans.

Class	Average animal usage in tonnes	Avg. human usage in tonnes
Aminoglycosides	12.46	0.84
Amphenicols	3.68	0.99
Carbapenems	-	3.86
Cephalosporins	5.77	3.56
Fluoroquinolones	8.97	81.65
Macrolides	21.70	138.90
Penicillins	73.04	2237.31
Polymyxins	16.19	20.00
Sulfonamides	32.34	109.07
Tetracyclines	107.38	19.11
Trimethoprim	4.77	37.77

$$\ln\left(Resistance_{i,t,g}\right) = \alpha + \beta \ln\left(Animal\_Use_{i,t}\right) + \gamma \ln\left(Human\_Use_{i,t}\right) + \delta \ln\left(Resistance_{i,t-1}\right) + \lambda_t + \boldsymbol{X_{i,t}} + \varepsilon_{i,t}$$

where $Resistance_{i,t,g}$ is the prevalence of resistance for unit for unit i, in year t, for "groups" g indicating either humans or food-producing animals. A unit i is formed by means of stratifying our data by country, bacteria, and antibiotic. The explanatory variables of interest in both the models are usage in food-producing animals and humans for unit i, in year t, denoted by $Animal\_Use_{i,t}$ and $Human\_Use_{i,t}$ respectively.

We include unit fixed effects in FE estimation, denoted by θ_i, to account for unobserved characteristics that are specific to units or different stratifications and are constant over time. Both estimation strategies include year (λ_t) fixed effects, which controls for unobserved variables that are specific to a particular year but shared across countries. In LDV estimation, instead of using unit fixed effects (θ_i) we use one-year lagged dependent variable denoted by $Resistance_{i,t-1}$. This strategy accounts for the fact that unobserved unit or group characteristics may not be fixed over time and, instead, past resistance values influence the current value of resistance. In other words, this model is designed to model past resistance as a time-varying confounder which cannot be controlled for by using fixed effects. $\boldsymbol{X_{i,t}}$ is a vector of country-and year-specific controls: population Correction Unit (PCU) and total human population. PCU for food-producing animals and total population of humans take into account difference in sizes and structure of the food-producing animal population and human population in each European country. We add Gross Domestic Product, health expenditure *per capita*, and the Corruption Perception Index as additional covariates in the sensitivity analysis section, but we do not anticipate these control variables to have a substantial effect on the coefficients of interest, given the use of country fixed effects or the lagged dependent variable. The error term is given by $\varepsilon_{i,t}$. Coefficient β measures the effect of antibiotic usage in food-producing animals on resistance. Similarly, coefficient γ measures the effect of antibiotic usage in humans on resistance. Both coefficients should be interpreted as elasticities, i.e., the percentage increase (decrease) in resistance correlated with a percentage increase (decrease) in usage. The models using resistance in humans and resistance in food-producing animals are estimated separately.

TABLE 2 Antibiotic resistance by bacteria in food-producing animals and humans.

Bacteria	Average resistance (%) in animals	Average resistance (%) in humans
Campylobacter	24.64	27.22
Escherichia	18.31	25.12
Salmonella	12.85	13.36

The use of the two specifications enables us to check the robustness of our findings using alternative identifying assumptions. That is, findings from both specifications should be broadly similar. Moreover, according to Angrist and Pischke (54), fixed effects and lagged dependent variable estimates have a useful bracketing property. As they show, the LDV specification provides the lower bracket while the FE specification provides the upper bracket. Thus, using these two specifications enables us to bound the causal effect.

Furthermore, we carry out sensitivity analysis of our findings based on these two specifications. First, to test for robustness of these results, we replicate these regressions after lagging the usage variables by 1 or 2 years. Second, we alter the sample definition, excluding outliers and including only specific bacteria. Third, we include covariates that have been shown to be related to antibiotic usage or resistance (7, 38, 55). Fourth, we ran these regressions after excluding small countries (those with a population under 6 million people) as a further robustness test. If our estimation strategy is sound, we anticipate that the results using different samples and additional covariates should not differ much from our main results.

4. Results

Table 1 presents average antibiotic usage, given in tonnes, by class. *Tetracyclines* are the most heavily used antibiotics class in food-producing animals while in humans, *Penicillins* are most used. Our data does not include *Carbapenem* use in animals. Given the high variation in use across different antibiotics, we tested exclusion of heavily and lightly used antibiotics, as described below.

As seen in Table 2, *Campylobacter* exhibits the highest average resistance in food-producing animals followed by *Escherichia coli* and *Salmonella*. In humans, *Campylobacter* also exhibits the highest resistance followed by *Escherichia coli*. The distribution plots and central tendency tables in Supplementary Tables S1, S2; Supplementary Figures S1–S4 show that the data on resistance and usage vary widely. Since we anticipate that the relationship between usage and resistance is most likely to be related to percentage changes, rather than unit changes, we log transform these variables. This also makes the range of the variables much more compact.

The correlations between log-transformed antibiotic usage and resistance measures for food-producing animals and humans generally show a positive relationship. Figure 2 presents the scatter plots along with the line of best fit showing the 95% confidence interval with all data pooled. We find strong positive correlations between usage and resistance for both animals and humans and between the two.

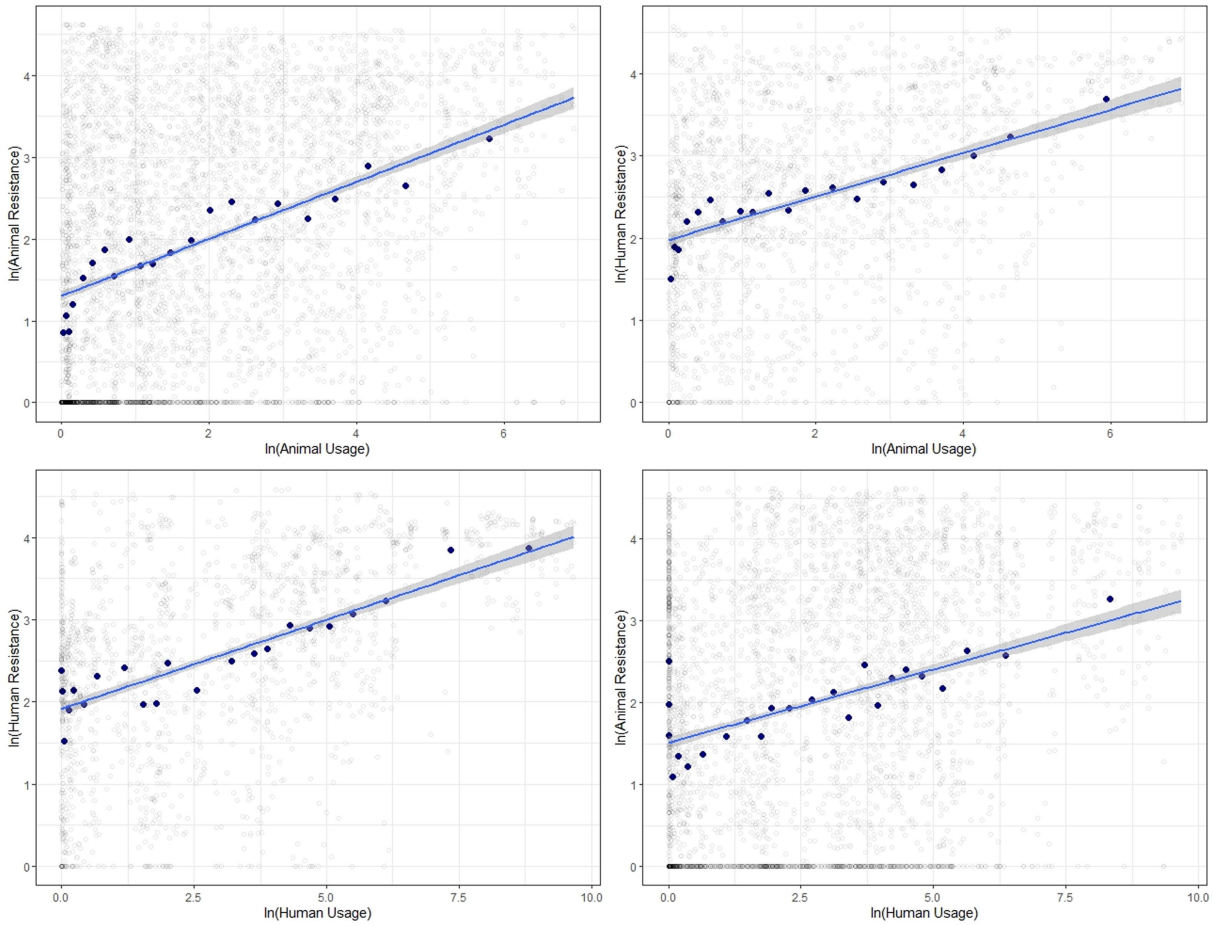

FIGURE 2
Binned scatter plots along with lines of best fit between animal usage and animal resistance (top-left), animal usage and human resistance (top-right), human usage and human resistance (bottom-left) and human usage and animal resistance (bottom-right). The light gray circles show the raw data.

We show similar plots with the data disaggregated by year (Figure 3), bacteria (Figure 4) and antibiotic class (Figure 5). The strength of these associations varies but we find positive correlations between usage and resistance across all years, bacteria and classes (except for a negative correlation between human usage and animal resistance for the two antibiotic classes, *Cephalosporins* and *Tetracyclines*). We go a step further and disaggregate these relationships by year and by bacteria for each country. Results are presented in Supplementary Figures S5, S6. Associations between resistance from animals and humans also exhibit positive correlations. The same is true for associations between usage from the two (Supplementary Figure S7).

These plots reveal positive correlations between antibiotic usage and the prevalence of resistance for a given year, country, bacteria and antibiotic class. The positive association exists within the same group and across groups. That is, usage of antibiotics in animals is positively related to occurrence of resistance in animals and in humans. Similarly, human usage of antibiotics is positively related to resistance levels in humans and in animals.

After exploring the correlations described above, we estimate the relationship between antibiotic usage and resistance using regression analysis. The results from our models are presented in Table 3. Columns 1 and 2 show estimated elasticities relating to resistance in animals, and columns 3 and 4 show those for resistance in humans. As described above, these relationships can be interpreted as indicating the lower and upper bounds of a causal effect. Columns 1 and 3 form the lower bounds for resistance effects and columns 2 and 4 form the upper bounds. We find that a 1% increase in antibiotic usage in animals increases resistance in animals between 0.22% and 0.41% and in humans between 0.03% and 0.40%. In addition, a 1% increase in antibiotic usage in humans leads to an increase in resistance in animals between 0.06% and 0.13% and in humans between 0.03% and 0.16%. All the coefficients, whether lower or upper bounds, are statistically significant. It is evident from these findings that an increase in antibiotic usage in both animals and humans contributes to an increase in resistance in animals and people.

The cross-species effect of usage on resistance is particularly important in this analysis. It is clear that higher usage can lead to higher resistance, but causality can also flow in the other direction, since with higher resistance, more antibiotics might be used to treat an infection. However, there is no direct mechanism by which higher prevalence of resistance in animals should lead to more intensive use of antibiotics in people. The cross-species effects are therefore naturally interpreted as being causal.

Table 4 shows the results of regressions in which human and animal use of antibiotics is summed, rather than being treated

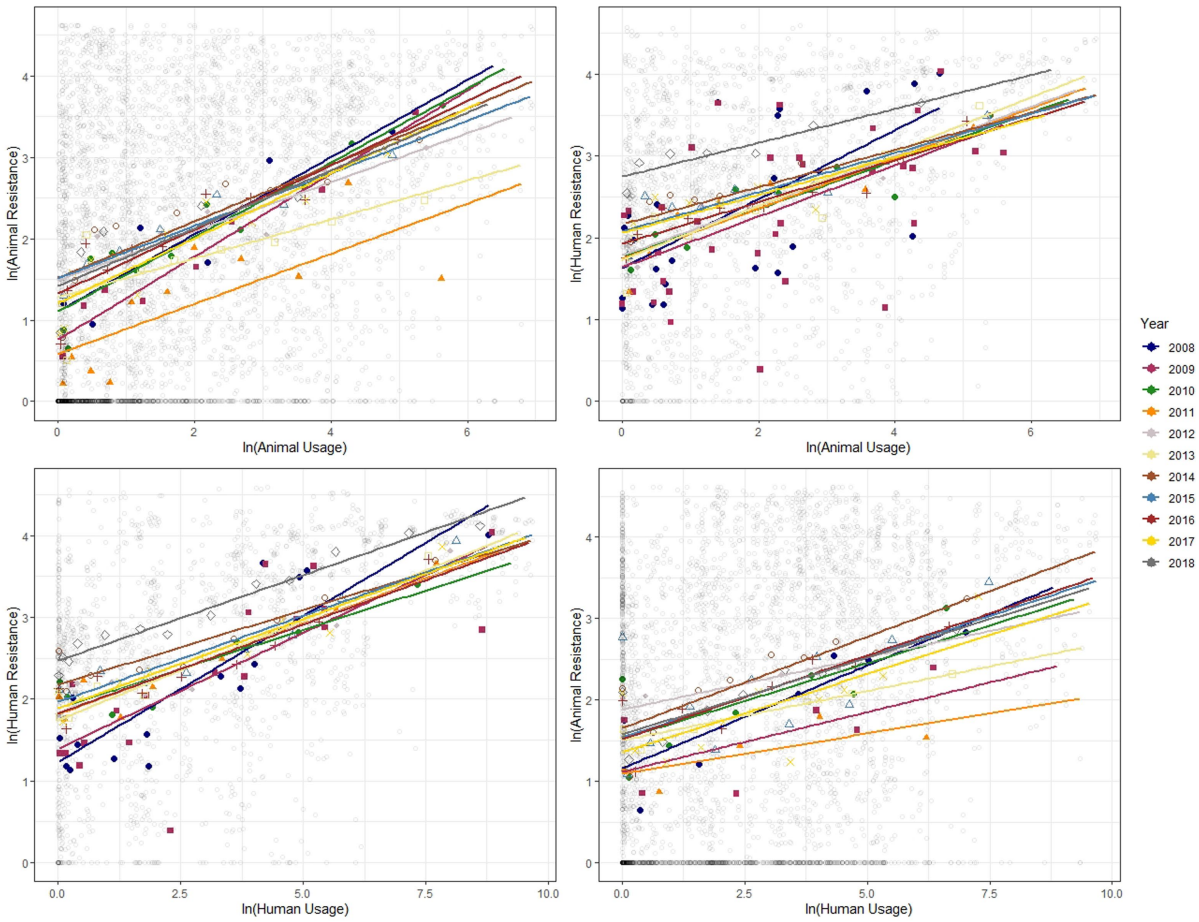

FIGURE 3
Binned scatter plots along with lines of best fit for years 2008–2018, between animal usage and animal resistance (top-left), animal usage and human resistance (top-right), human usage and human resistance (bottom-left) and human usage and animal resistance (bottom-right). The light gray circles show the raw data.

separately. We find consistent results. Generally, the upper and lower bounds are somewhat tighter in these regressions.

To further investigate the causal effects, we go a step further. Instead of using contemporaneous usage we lag the usage variables by 1 and 2 periods. This addresses the problem of reverse causality, since resistance in the current year should not have any effect on usage in preceding years. The results are presented in Table 5. Columns 1–2 and 5–6 display the bounded effect on animal and human resistance respectively, from usages lagged by one year. We find strong and statistically significant evidence of usage driving resistance across and within humans and food-producing animals. The results are quite similar when the usage variables are lagged 2 years as shown in columns 3–4 and columns 7–8. Lagging the usage variables results in estimated coefficients that are broadly in line with the contemporaneous usage data.

4.1. Further robustness checks

To check for robustness of our results we estimate both models after excluding possible outliers. We exclude penicillin, aminoglycosides and amphenicols separately from our data and run the models. As shown in Table 1, penicillin is the most used antibiotic class in humans and aminoglycosides and amphenicols are the least used classes. Therefore, we treat them as outliers and exclude them separately from our data. In addition, we also run the models separately for individual bacteria, by including additional control variables and by excluding countries with a population less than 6 million people. The results for this sensitivity analysis are presented in Supplementary Tables S3–S6.

Sensitivity analysis reveals our results are robust to different sample definitions. When the sample size is restricted by including only one bacterial species at a time, the statistical significance is naturally lower. The effects are always statistically significant for the fixed effect estimates, but not necessarily for the LDV specification. The range of resistance effects when we exclude countries with populations less than 6 million people are also robust and close to estimates in Tables 3, 4.

5. Discussion

The inherent convolution of growing antimicrobial resistance makes it difficult to understand the exact ways in which it spreads

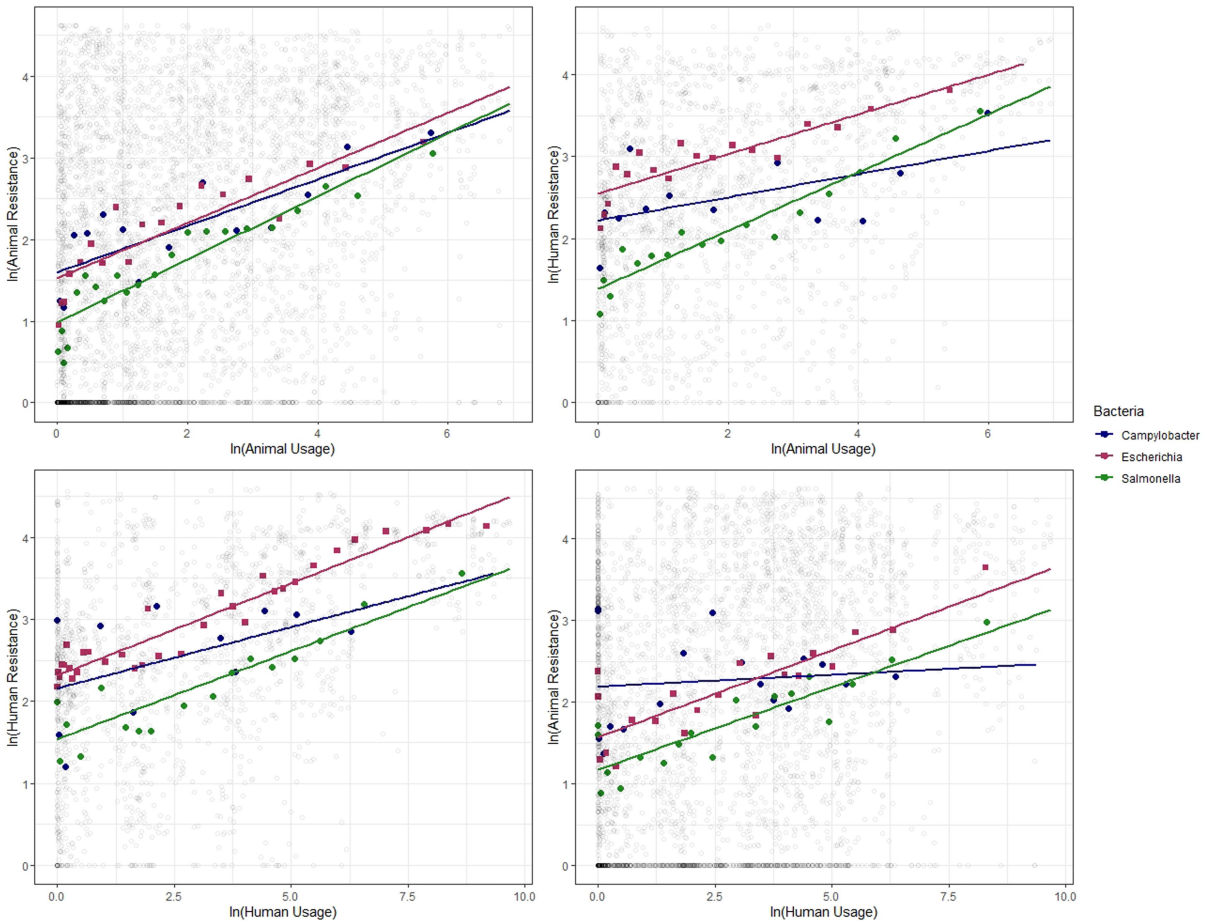

FIGURE 4
Binned scatter plots along with lines of best fit for the 3 bacteria genera, between animal usage and animal resistance (top-left), animal usage and human resistance (top-right), human usage and human resistance (bottom-left) and human usage and animal resistance (bottom-right). The light gray circles show the raw data.

among domesticated animals, humans, and the environment. This paper aims to shed light on the relationships between usage and resistance between and across human and food-producing animal populations in Europe. It highlights relationships that have not been covered extensively in the existing literature, specifically, the effect of usage in animals on human resistance and the effect of usage in humans on animal resistance. Using data from European surveillance reports, the paper shows that simultaneous and combined antibiotic usage in food-producing animals and humans have a positive impact on the incidence of resistance in both the populations.

What is novel in our analysis is that we simultaneously include both human and animal use of antibiotics when examining the relationships with resistance. This enables us to observe the marginal relationship between antibiotic use and resistance in humans and animals separately, conditional on use in the other group. Moreover, our econometric approach enables us to infer causality at an ecological level. That greater use of antibiotics would increase the prevalence of resistance is of course not surprising: however, we are able to quantify this effect within a range. In addition, we are able to show for the first time the effect of human use of antibiotics on the prevalence of resistance in food-producing animals.

The estimated effects are both substantial and statistically significant. Strikingly, the lower and upper bounds of the effect of antibiotic use in animals on resistance in humans are not smaller than the effect of antibiotic use in humans. The estimated elasticities are, from the perspective of long-term impact on resistance, disturbingly large. Even at the lower bound, an increase in antibiotic use in animals of only 10% is expected to increase the prevalence of resistance in animals by around 2% and in humans by around 0.3%. Since, as we show in a recent paper, resistance tends to persist over a period of years, increases in usage may lead to long-term increases in resistance (42).

Our study has numerous limitations. We are only able to provide the average effects of usage on resistance for Europe as a nation. Our data do not allow us to explicate clear mechanisms of how usage affects resistance within or across human and food-producing animal populations. There are several factors at play that determine the occurrence of resistance against antibiotics among bacteria. This includes the ancient molecular mechanisms behind the emergence of resistant bacteria and the natural concentration of antibiotics and resistant genes in the environment (56–58). It would be ideal to consider all the factors that contribute to the evolution of antibiotic resistance in bacteria; however, appropriate measures for the impact

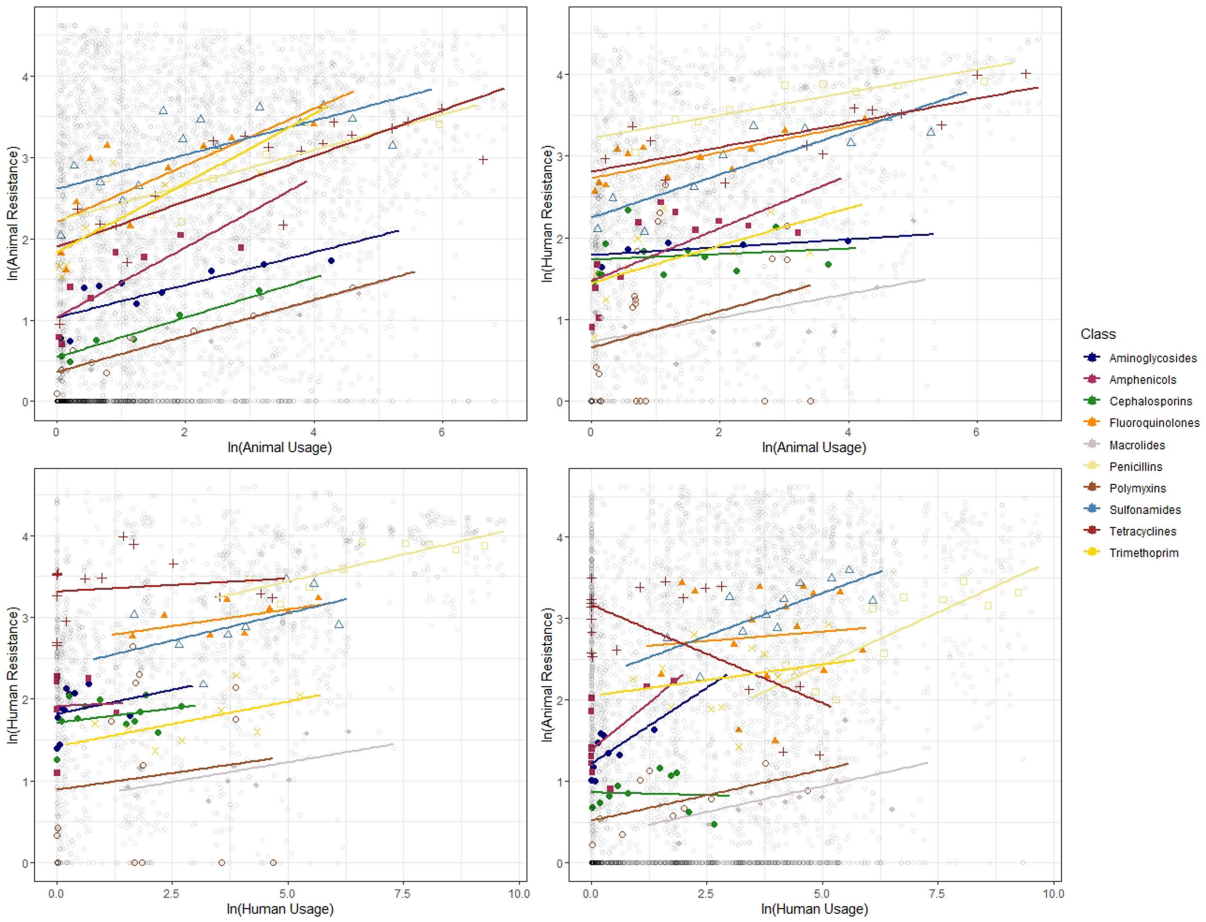

FIGURE 5
Binned scatter plots along with lines of best fit for the 11 antibiotic classes, between animal usage and animal resistance (top-left), animal usage and human resistance (top-right), human usage and human resistance (bottom-left) and human usage and animal resistance (bottom-right). The light gray circles show the raw data.

TABLE 3 Simultaneous usage effects of antibiotics on animal and human resistance.

	(1)	(2)	(3)	(4)
Variables	*ln* (animal resistance)		*ln* (human resistance)	
ln (animal usage)	0.224*** (0.0178)	0.408*** (0.0205)	0.0306** (0.0117)	0.397*** (0.0230)
ln (human usage)	0.0647*** (0.00874)	0.133*** (0.00903)	0.0283*** (0.00600)	0.157*** (0.00896)
Constant	1.001 (0.577)	35.61 (23.99)	0.836** (0.318)	73.02** (28.02)
Observations	3,062	3,983	1,562	1818
R-squared	0.486	0.273	0.800	0.364
Year FE	YES	YES	YES	YES
Country FE	NO	YES	NO	YES
Lagged dependent variable	YES	NO	YES	NO

Standard errors in parentheses *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

of environmental factors are limited and there is a disconnect in surveillance data for humans and animals (59, 60).

Antibiotic usage has been linked to an increase in resistance in both humans and food-producing animals, making it a critical public health issue. Injudicious use and over-use of antibiotics within and across clinical and agricultural settings provides a favorable environment for the emergence of antibiotic resistant bacteria, causing infections that are difficult to treat (61). The potential health implications of these are significant, as antibiotic resistant bacteria can spread from food-producing animals to

TABLE 4 Combined usage effects of antibiotics on animal and human resistance.

	(1)	(2)	(3)	(4)
Variables	*ln* (animal resistance)		*ln* (human resistance)	
ln (combined usage)	0.139*** (0.0109)	0.274*** (0.00950)	0.0420*** (0.00782)	0.296*** (0.00887)
Constant	−0.374 (0.542)	43.64 (24.02)	0.800** (0.292)	76.38** (27.30)
Observations	3,062	3,983	1,562	1818
R-squared	0.479	0.271	0.800	0.367
Year FE	YES	YES	YES	YES
Country FE	NO	YES	NO	YES
Lagged dependent variable	YES	NO	YES	NO

Standard errors in parentheses *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

TABLE 5 Simultaneous effects of lagged antibiotic usage on animal and human resistance.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Variables	*ln* (animal resistance)				*ln* (human resistance)			
ln (animal usage)	0.188*** (0.0178)	0.414*** (0.0215)	0.280*** (0.0226)	0.425*** (0.0227)	0.0366* (0.0161)	0.386*** (0.0256)	0.0569** (0.0192)	0.399*** (0.0262)
ln (human usage)	0.0610*** (0.00853)	0.147*** (0.00961)	0.105*** (0.0111)	0.146*** (0.0102)	0.0417*** (0.00756)	0.160*** (0.00980)	0.0585*** (0.00868)	0.161*** (0.0102)
Constant	1.656** (0.527)	31.82 (31.69)	1.190 (0.742)	−15.25 (39.59)	1.068* (0.417)	90.18** (33.26)	1.380** (0.512)	117.1** (44.11)
Observations	2,325	3,046	1,828	2,568	1,277	1,572	1,012	1,321
R-squared	0.615	0.310	0.446	0.298	0.745	0.358	0.734	0.377
Year FE	YES	YES	YES	YES	YES	YES	YES	YES
Country FE	NO	YES	NO	YES	NO	YES	NO	YES
Lagged dependent variable	YES	NO	YES	NO	YES	NO	YES	NO
Usage variables lagged 1 year	YES	YES	NO	NO	YES	YES	NO	NO
Usage variables lagged 2 years	NO	NO	YES	YES	NO	NO	YES	YES

Standard errors in parentheses *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

humans and *vice-versa*, resulting in increased morbidity and mortality (62, 63). In light of this matter, policymakers have a crucial role to play in addressing antibiotic resistance. A recent study projects the use of antibiotics in animal farming to increase by 8% between 2020 and 2030 (64). One immediate measure is to implement policies aimed at curbing and promoting judicious use of antibiotics in human medicine and animal production. However, as we show in a previous study, decreasing antibiotics use alone may not be a sufficient solution (42). Along with judicious antibiotic use, development of alternative technologies, including using innovative financial mechanisms such as the UK's antibiotic subscription pilot may be necessary (65, 66). Moreover, policies to encourage farmers and healthcare providers to adopt preventive measures, such as improved hygiene and vaccination, could potentially reduce the need for antibiotics and mitigate the development of resistance (67). Therefore, addressing this issue requires a multi-disciplinary approach that involves stakeholders from all relevant sectors and recognizing the health of animals, humans and the environment are interwoven.

It is notable that this study's data are drawn from European countries, which tend to have relatively low rates of antibiotic usage and resistance (68). Since antibiotic resistance is a more pressing problem in many low-and middle-income countries, it would be useful to better understand the relationship between usage and resistance in those countries (69, 70). Understanding the effect of antibiotic consumption on rates of resistance is of great importance and will require ongoing investment into consistent surveillance data on a global scale.

6. Conclusion

To summarize, this paper provides new insights into the complex relationships between antibiotic usage and resistance in

humans and food-producing animal populations in Europe. Our analysis reveals that usage of antibiotics by both humans and food-producing animals has a significant and statistically relevant effect on the rates of resistance in both groups. The estimated own-and cross-elasticities are worrying and highlight the potential long-term impacts of antibiotic usage on resistance. However, the study has limitations, including the lack of clear mechanisms explaining the relationship between usage and resistance and the inability to account for environmental factors. Antibiotic resistance is a critical public health concern, and policymakers need to promptly adapt a multi-disciplinary approach which engages all relevant stakeholders and acknowledges the interdependence of animal, human and environmental health. Simultaneous usage of antibiotics in various sectors and direct and indirect sharing of resistance across humans, animals and environment calls for a need to implement integrated strategies to monitor usage and resistance across heterogenous One Health dominions.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found at: PRISM Data: University of Calgary's Data Repository—Replication Data for: the effect of antibiotic usage on resistance in humans and food-producing animals: a longitudinal, One Health analysis using European data, doi: 10.5683/SP3/RXHWFP.

Author contributions

SR and AH: conceptualization, data verification, investigation, methodology, writing, and review and edit. SR: literature review, data curation, and formal analysis. AH: funding acquisition and supervision. 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/fpubh.2023.1170426/full#supplementary-material

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Epidemiology of companion animal AMR in the United States of America: filling a gap in the one health approach

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Introduction: Antimicrobial resistance (AMR) is a global health concern that affects all aspects of the One Health Triad, including human, animal, and environmental health. Companion animals, such as cats and dogs, may contribute to the spread of AMR through their close contact with humans and the frequent prescription of antimicrobials. However, research on AMR in companion animals is limited, and there are few surveillance measures in place to monitor the spread of resistant pathogens in the United States.

Methods: This study aims to explore the practicality of using data from commercial laboratory antimicrobial susceptibility testing (AST) services for epidemiological analyses of AMR in companion animals in the United States.

Results: The study analyzed 25,147,300 individual AST results from cats and dogs submitted to a large commercial diagnostic laboratory in the United States between 2019 and 2021, and found that resistance to certain antimicrobials was common in both *E. coli* and *S. pseudintermedius* strains.

Conclusion: There has been a paucity of information regarding AMR in companion animals in comparison to human, environmental and other animal species. Commercial AST datasets may prove beneficial in providing more representation to companion animals within the One Health framework for AMR.

KEYWORDS

antimicrobial resistance, *E. coli*, *S. pseudintermedius*, epidemiology, companion animal

1. Introduction

Antimicrobial resistance (AMR) is a primary global health concern, affecting all fractions of the One Health Triad: human, animal, and environmental health (1). Resistance can also be transferable between human and animal species, making AMR a zoonotic health concern as well (2, 3). Articles stressing the importance of a One Health approach to AMR often neglect the role of companion animals, failing to account for a missing piece of the puzzle.

Growing awareness of the potential contribution of companion animals to the spread of resistant pathogens has led to a greater research focus on household cats and dogs due to the intimate bond shared with their owners. Frequent close contacts present the opportunity for

transmission of antimicrobial resistant bacteria (4), but are rooted in a deeply held bond owners share with their animals (5). Additionally, companion animals share more antimicrobials and are treated for infections in a manner more similar to humans than livestock animals, presenting greater opportunity for resistant pathogen spillover (6). However, data on companion animal AMR are typically less abundant than that of production animals. The American Food and Drug Administration recently recognized the absence of research into companion animal AMR and the immediate need to fill this gap in the One Health approach (7).

In the United States, it is estimated that one in five households acquired a new cat or dog during the COVID-19 pandemic (8), adding to the previous 135 million pets owned in 2018 (9). As this new generation of cats and dogs age, there will be an inevitable demand for antimicrobial treatments. Coupled with current resistance concerns, this demand may lead to the propagation of new and existing strains of resistant microbes within the next 15 years, for which surveillance measures must be put into place. For such surveillance, sufficient data must be collected continuously over a broad geographic area. Routine susceptibility testing by diagnostics laboratories may provide an avenue to achieve this level of data collection, but the usefulness of such data sources for epidemiological studies of companion animal AMR has yet to be adequately assessed due to limited access. Previous works have leveraged electronic health records from companion animals to develop a passive surveillance system for tick monitoring (10), and such methodologies may be applicable for AMR surveillance, but first data sources must be explored.

An estimate of the global burden of antimicrobial resistance in 2019 found over 5 million human deaths attributable to AMR, 75% of them being accounted for by only six pathogens, with *Escherichia coli* and *Staphylococcus aureus* being at the top of the list (11). Resistant *S. aureus* represents a major health concern for humans but is not as commonly isolated in cats and dogs compared to *S. pseudintermedius*, which can commonly be misidentified as *S. aureus* in human animal bites (12). *S. pseudintermedius* is an underreported issue in human medicine, but awareness is increasing due to its similarities to other human pathogens (13, 14).

This study aims to highlight the underrepresentation of companion animals in the current One Health framework of antimicrobial resistance. Companion animals are often neglected from public health reports on AMR due to the inaccessibility of data in comparison to humans, and livestock (15). However, routine commercial antimicrobial susceptibility testing may offer a wide-reaching and continuing source of data for epidemiology and surveillance. This study demonstrates the application of these types of data. Given the complexity of antimicrobial resistance, it is not feasible to represent all drug-pathogen resistance combinations in a single study. Therefore, a selection of clinically relevant *E. coli* and *S. pseudintermedius* resistance concerns are investigated, to provide a high-level overview of the status of known and emerging resistance concerns, using a previously unexploited source of data.

2. Materials and methods

Samples from cats and dogs submitted to a nation-wide commercial diagnostic laboratory within the United States for bacteriological testing and subsequent antimicrobial susceptibility testing between 2019 and 2021 were eligible for inclusion. Each observation was recorded with the following information: a unique deidentified patient number, the date of sample collection (down to monthly accuracy), the location of the submitting veterinary practice (three-digit ZIP code accuracy), the source from which the sample was taken on the animal, the identified pathogen isolated in the sample, and the antimicrobial susceptibility status for all drugs tested against the isolated pathogen. Susceptibility status was reported as either "susceptible," "resistant," or "intermediate" based on clinical breakpoint values set forth by the Clinical and Laboratory Standards Institute (CLSI) (16). Duplicated samples were defined as possessing the same identification number, same isolated pathogen, occurring within the same three-month quarter and the same susceptible/intermediate/resistant status. Duplicates were removed. All susceptibility testing was conducted on the VITEK (bioMérieux) automated platform (17). Where VITEK testing was not possible, the Kirby-Bauer method was used (18). Canine and feline data were assessed in aggregate.

Four drug-pathogen susceptibility scenarios were investigated, including two representing common first line prescription approaches and two emerging resistance concerns of higher priority drugs. The specific drug-pathogen combinations included: amoxicillin resistant *Escherichia coli* isolated from urine samples, cephalexin (first-generation cephalosporin) resistant *S. pseudintermedius* isolated from skin samples, third-generation cephalosporin resistant *E. coli* (any source), and methicillin resistant *S. pseudintermedius* (any source). Third-generation cephalosporin resistance was indicated primarily by cefotaxime resistance. Where cefotaxime was not tested but another third-generation cephalosporin was (i.e., ceftazidime or cefpodoxime), that antimicrobial would be included in its place, with a limit of one drug per sample. Oxacillin was used to test for methicillin resistance.

Choropleth maps representing the percentage of samples found to be non-susceptible were produced for each of the four drug-pathogen combination scenarios at the state level. Non-susceptibility was defined as a sample either being reported as resistant or intermediately resistant. State borders were acquired from the '*usmaps*' package in the statistical software *R* (19, 20). Where applicable, Wald 95% confidence intervals were used given the large sample size. The data were then further stratified into individual calendar years, and relative risk values for each state were calculated using the following formula: proportion of resistant samples within the state divided by the proportion of resistant samples in all the United States for a given year. This metric provided a standardized measure to indicate the deviation of a given state from the nationally expected amount of resistance, with values less than 1 indicating less than expected resistance, values greater than 1 indicating greater than expected resistance and a value of 1 indicating equal to the expected resistance. These relative risk values were mapped and arranged in chronological order for each drug-pathogen scenario to provide indication of any emerging temporal patterns. In both maps, information on any state with less than 30 sample observations was censored as per CLSI's report 'Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data, 5th Edition' (21).

To compare the level of resistance between drugs of the same class against a common pathogen, the proportion of susceptible, resistant and intermediate interpretations were tabulated for all the tested third generation cephalosporins and fluoroquinolones against *E. coli* or *Staphylococcus* spp. (*S. aureus, S. intermedius, S. pseudintermedius,*

S. schleiferi). All samples of *E. coli* or *Staphylococcus spp.* submitted for susceptibility testing were included, regardless of repeated testing. These tabulated proportions of test interpretations provide an opportunity to check for differences in reported susceptibility in instances where high agreement should be present.

To further explore how observed susceptibility compares between various antimicrobials against the same submitted sample, an agreement matrix was produced showing the proportion of instances where pairs of drugs arrived at the same susceptibility interpretation. For this matrix, susceptibility was dichotomized to susceptible and non-susceptible (resistant and intermediate combined). Only samples tested against the complete list of drugs in the matrix were included. This list of drugs was compiled based on the most frequent panel tested for the given pathogen and source observed in the data. All drugs in the panel were matched pairwise and the proportion of times the same susceptibility interpretation was observed was recorded in the matrix. A proportion of 1 would indicate that on every sample tested the two drugs always resulted in the same dichotomized susceptibility interpretation. Two agreement matrices were produced, one for *E. coli* isolated from urine samples and one for *S. pseudintermedius* isolated from skin samples.

3. Results

The dataset was comprised of 25,147,300 single antimicrobial susceptibility tests performed on 1,295,480 isolates submitted for 760,157 individual patients. A breakdown of high-level descriptive statistics is displayed in Table 1.

3.1. Amoxicillin resistant *Escherichia coli* (urinary isolates)

Overall, 67.0% (66.8, 67.1%) of *E. coli* were not susceptible to amoxicillin. The lowest percentage of susceptible samples across the three-year study period were observed in Louisiana [56.9% (54.4, 59.5%)] and Alabama [57.0% (53.3, 60.5%)]. The state with the greatest observed susceptibility was Montana [79.4% (75.2, 83.7%)]. A state breakdown of the observed non-susceptible samples can be seen in Figure 1. In general, states in the southeast appear to have observed the greatest proportion of non-susceptible samples of *E. coli* to amoxicillin. Observing the relative risks of each state for each of the 3 years shows relative temporal stability in the distribution of amoxicillin resistant *E. coli* (Figure 2). The southeastern states were continuously observed to have the greatest relative risk each year, in contrast to the western and Midwest states who showed continuous relative risk values at or below one. The range of relative risk (0.54–1.39) indicates a moderate discrepancy in risk across the country.

3.2. First-generation cephalosporin resistant *S. pseudintermedius* (skin isolates)

Overall, there was a prominent level of first-generation cephalosporin resistant *S. pseudintermedius* observed across the country, with only 58.1% (57.7, 58.4%) of samples found to be susceptible. Susceptibility as low as 48.8% (47.5, 50.1%) was observed in Texas (Figure 3). Two states, Wyoming and North Dakota, failed to reach the minimum sample size to be included in the map. Mapped relative risks showed a relatively even distribution of risk across the country and stability over the 3 years studied. No clear spatial trend could be concluded through visual analysis (Figure 4).

3.3. Third-generation cephalosporin resistant *Escherichia coli* (All sources)

E. coli isolated and tested during the study period were found to be highly susceptible to third generation cephalosporins (cefotaxime, ceftazidime, and cefpodoxime), at 83.5% susceptibility (83.3, 83.6%) across the country (Figure 5). Despite the overall high susceptibility, considerable variability in relative risk was observed, where the southeastern states (Alabama, Florida, Louisiana, Mississippi, and Texas) were up to 1.86 times more likely to observe a resistant sample than the national average (Figure 6). Visual analysis of the relative risk maps revealed a clear spatial pattern of elevated risk in the southeastern states with diminishing risk moving to the northwest.

3.4. Methicillin resistant *S. pseudintermedius* (All sources)

Methicillin resistant *S. pseudintermedius* was observed across the country, with a national susceptibility rate of 67.3% (67.1, 67.4%). The lowest susceptibility was observed in Texas [57.3% (56.6, 58.2%)] and the highest in Wyoming [77.9% (69.1, 86.7%)]. Similar spatial patterning was observed to the previous scenarios examined, with a greater level of resistance in the southeastern states and Texas, and lower levels of resistance moving to the northwest (Figure 7). Examination of the relative risk maps provide more evidence for this spatial trend and saw consistent distribution patterns over all 3 years (Figure 8). Relative risk values ranged from 0.46, in Rhode Island, to 1.42, in Texas.

3.5. Drug susceptibility agreement

A breakdown of susceptibility test results for both *E. coli* and *S. pseudintermedius*, against all drugs tested in the third generation cephalosporin and fluoroquinolone classes is presented in Table 2. Two drugs, cefotaxime and ceftiofur failed to meet the minimum sample size requirement to be included. Pradofloxacin, although meeting the CLSI recommended minimum sample size, was removed from the table for *E. coli* due to substantially fewer observations than the other drugs in the same class. Ceftazidime was removed for the same reason for *S. pseudintermedius*. All drugs within the same class were found to possess reasonably similar proportions of susceptible, intermediate and resistant observations (Table 2). The agreement matrices for *E. coli* and *S. pseudintermedius* susceptibility against commonly tested drugs are presented in Figures 9, 10, respectively. A high level of intra class agreement was observed in the matrices. Likewise, drugs acting through similar biological mechanisms showed a high level of agreement, as anticipated.

TABLE 1 Descriptive breakdown of data used in assessing resistance.

		E. coli		*S. pseudintermedius*	
		Amoxicillin (Urine[1])	Cephalosporin (III)	Cephalosporin (I) (Skin[2])	Methicillin/ Oxacillin
Sample Size (*n*)					
	2019	57,466	94,027	21,404	68,761
	2020	66,537	105,645	23,364	79,846
	2021	71,440	115,367	27,243	92,888
n by Species					
	Canine	145,156	243,261	70,985	234,667
	Feline	50,287	71,778	1,026	6,828
n by State (All years)					
	Minimum	143 (WY)	238 (WY)	13 (WY)	100 (WY)
	Maximum	44,756 (CA)	66,895 (CA)	12,182 (CA)	43,277 (CA)
	Median	1,582	2,582	625	2,036
	n < 30	0	0	2	0
% Non-susceptible by Species					
	Canine	27.8%	17.9%	41.7%	32.4%
	Feline	28.0%	11.2%	46.4%	43.8%
% Non-susceptible by State					
	Minimum	20.5% (MO)	8.58% (WY)	30.8% (MN)	22.1% (WY)
	Maximum	43.1% (LA)	28.1% (LA)	51.1% (TX)	42.6% (TX)
	National average	33.0%	16.5%	41.9%	32.7%
Relative risk					
	Minimum	0.54 [MO (2021)]	0.41 [ND (2020)]	0.34 [RI (2019)]	0.46 [RI (2019)]
	Maximum	1.39 [AL (2021)]	1.86 [MS (2020)]	1.35 [TX (2019)]	1.42 [TX (2019)]

[1]Urinary samples defined as urine collected by means of catheter, cystocentesis or free catch.
[2]Skin samples defined as samples classified as dermatitis, skin or skin tissue.

4. Discussion

The analysis performed in this study, while not comprehensive, offers foundational knowledge regarding the applications of laboratory susceptibility testing data, in addition to the status of several frequently referenced problems of concern in companion animal AMR. While there have been studies on companion animal AMR elsewhere, there has been a paucity of research in the United States (22–24). Many of the presented estimates of the national resistance burden are among the earliest to be reported at a national level for the United States, offering a unique opportunity to compare observed resistance between states under near identical submission and testing conditions. These results found similar spatial patterns of resistance across three of the four drug-pathogen combinations explored, wherein the southeastern states, and Texas were found to have higher than average levels of non-susceptible isolates. Conversely, the more northern states, particularly those to the west, were more likely to see higher levels of susceptibility. This pattern may reveal a spatial trend which should be further assessed to determine causality. In some scenarios (third generation cephalosporin resistant *E. coli* in particular) this pattern was quite pronounced with a range in relative risk of 0.41 in North Dakota to 1.86 in Mississippi, indicating a near 150% difference in risk between the northern and southern states. This pattern indicates that some outside factor of resistance is likely to be present, be it environmental (due to differing climates), cultural (differences in antimicrobial treatment practices), or systematic (a difference in how clinicians are deciding to submit a sample for testing).

Amoxicillin is a common first line drug for sporadic bacterial cystitis in companion animals (25). Similarly, cephalexin (a first-generation cephalosporin) is a commonly prescribed first line drug for the treatment of *S. pseudintermedius* and other skin and soft tissue infections (26). At a national level, resistance to amoxicillin in *E. coli* (urine) and cephalexin in *S. pseudintermedius* (skin) was observed in 33.0 and 41.9% of samples submitted, respectively. These results suggest that a large amount of common urinary and skin infections are resistant to frequently administered first line antimicrobials. Resistance to these first line treatments is a documented concern (27, 28), with susceptibility to amoxicillin in *E. coli* as low as 53% being reported in previous lab datasets in Kansas, United States (29). Resistance estimates may be overinflated in this and other laboratory datasets, as discussed subsequently in more detail. Patients included in antimicrobial susceptibility testing (AST) datasets are more likely to have failed first line treatments, thus warranting testing to find a suitable replacement. However, the general spatial patterning should remain representative, where resistance appears to be more prevalent

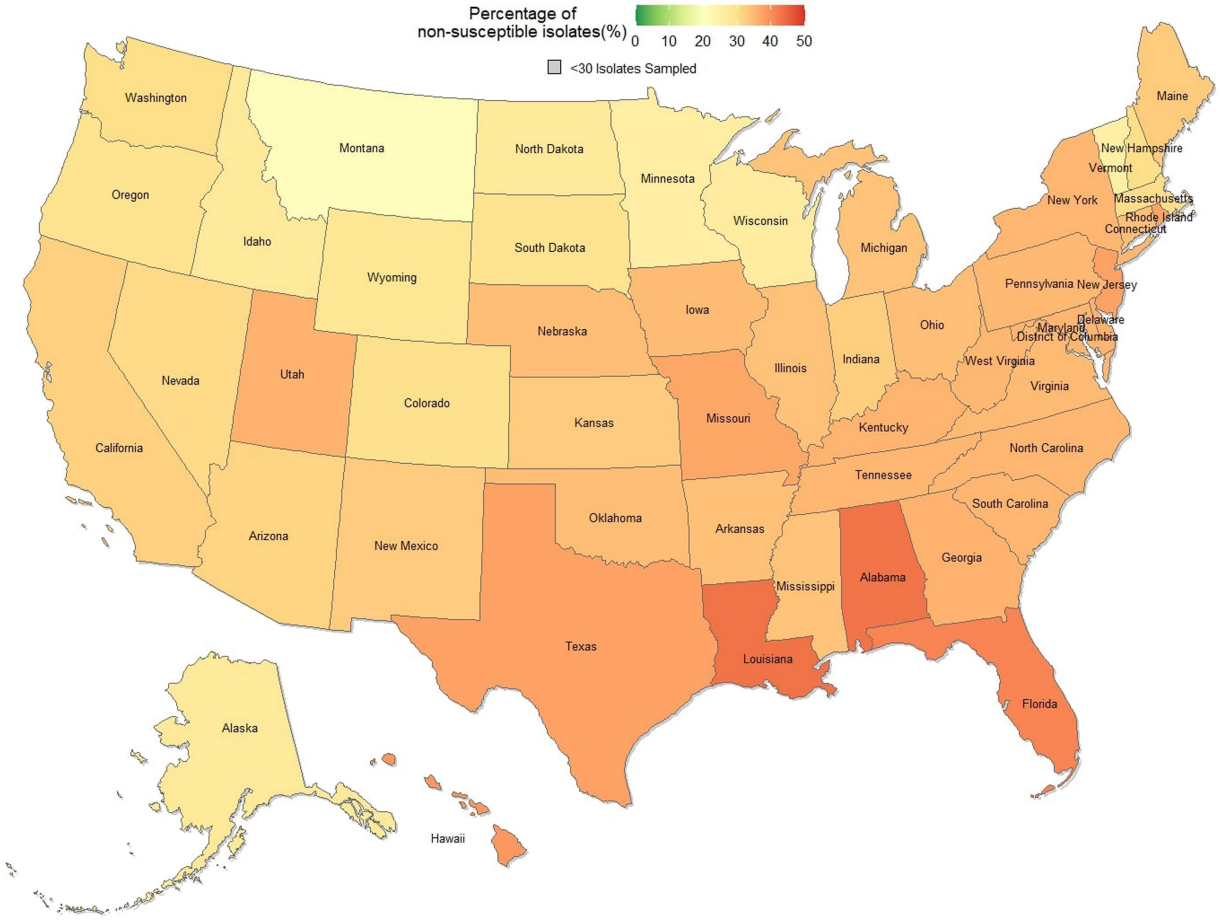

FIGURE 1
Map of the United States, depicting the percentage of *E. coli* isolates, from urinary tract infections in cats and dogs, found to be resistant to amoxicillin between 2019 and 2021.

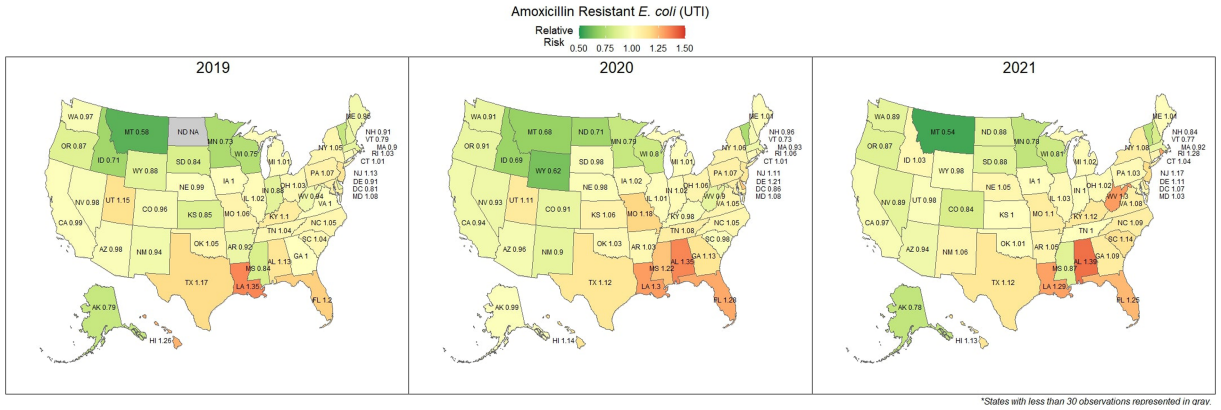

FIGURE 2
Maps of the United States, depicting the relative risks of amoxicillin resistant *E. coli*, from urinary tract infections in cats and dogs observed in 2019, 2020, and 2021.

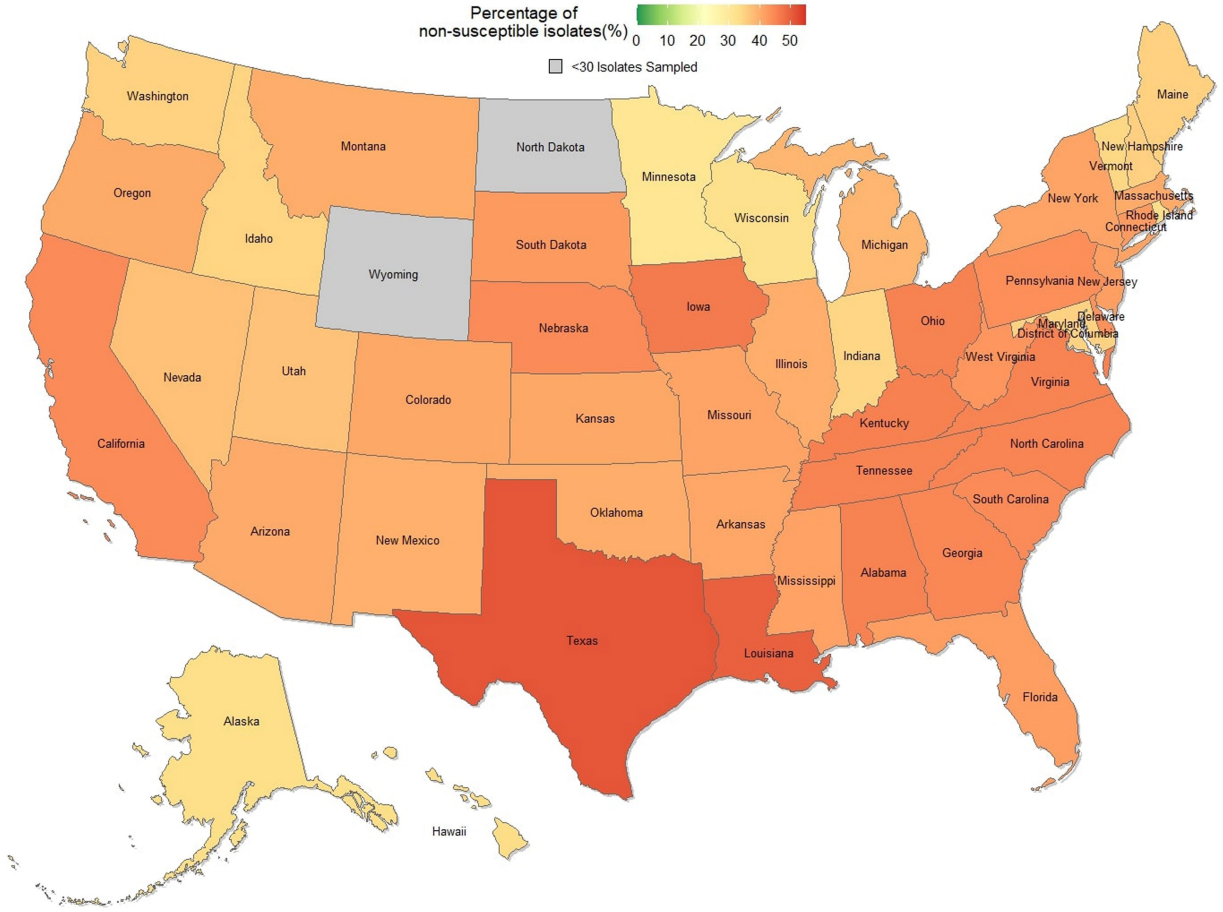

FIGURE 3
Map of the United States, depicting the percentage of *S. pseudintermedius* isolates, from skin infections in cats and dogs, found to be resistant to cephalexin between 2019 and 2021.

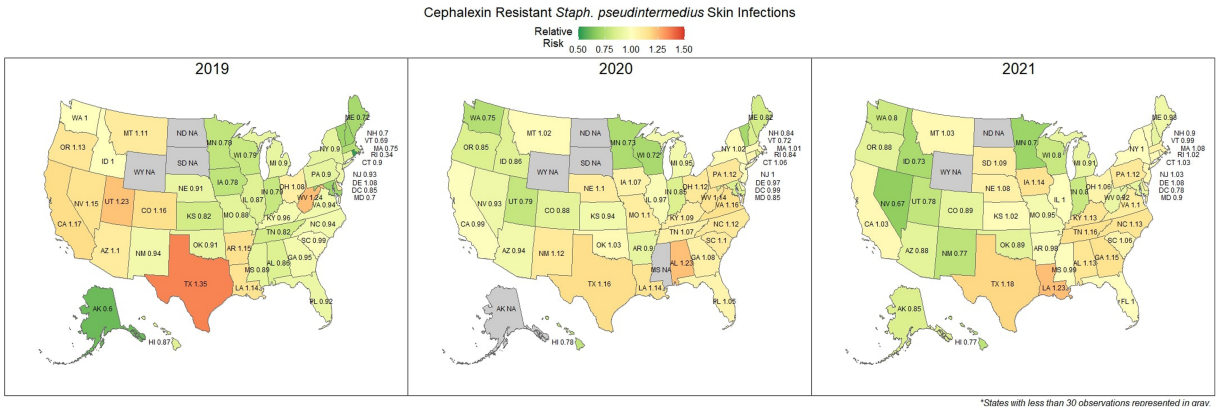

FIGURE 4
Maps of the United States, depicting the relative risks of cephalexin resistant *S. pseudintermedius*, from skin infections in cats and dogs observed in 2019, 2020, and 2021.

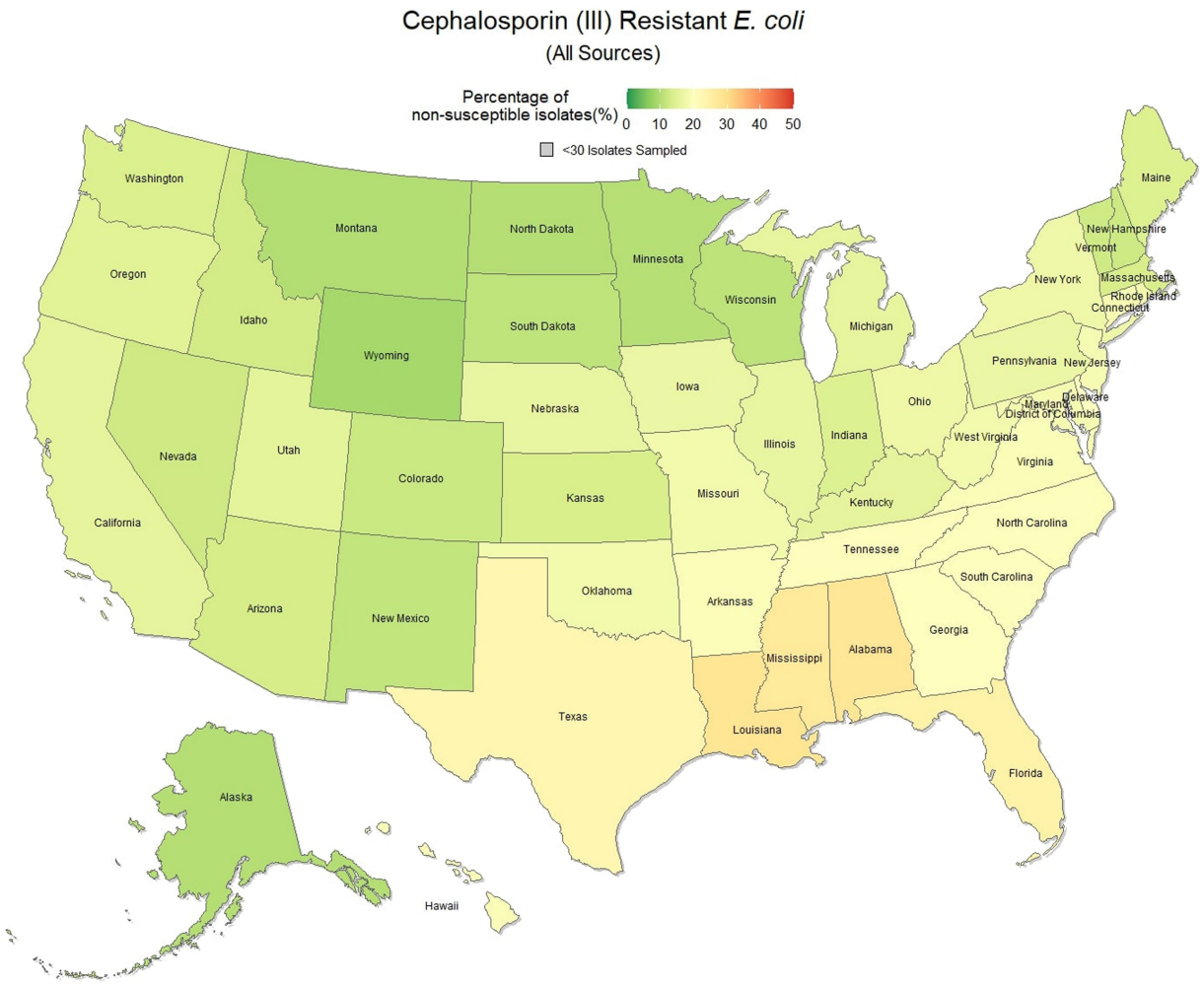

FIGURE 5
Map of the United States, depicting the percentage of *E. coli* isolates from cats and dogs found to be resistant to cephalosporins (III) between 2019 and 2021.

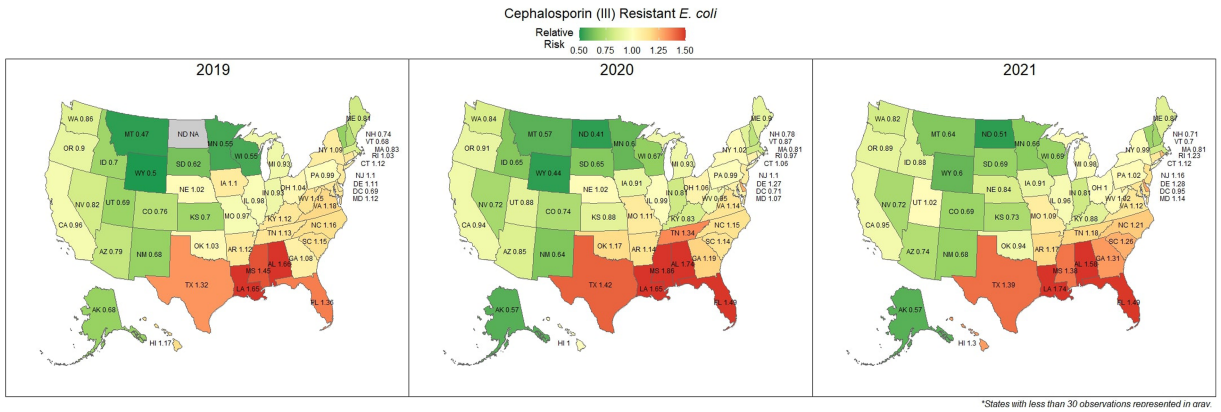

FIGURE 6
Maps of the United States, depicting the relative risks of cephalosporin (III) resistant *E. coli* infections in cats and dogs observed in 2019, 2020, and 2021.

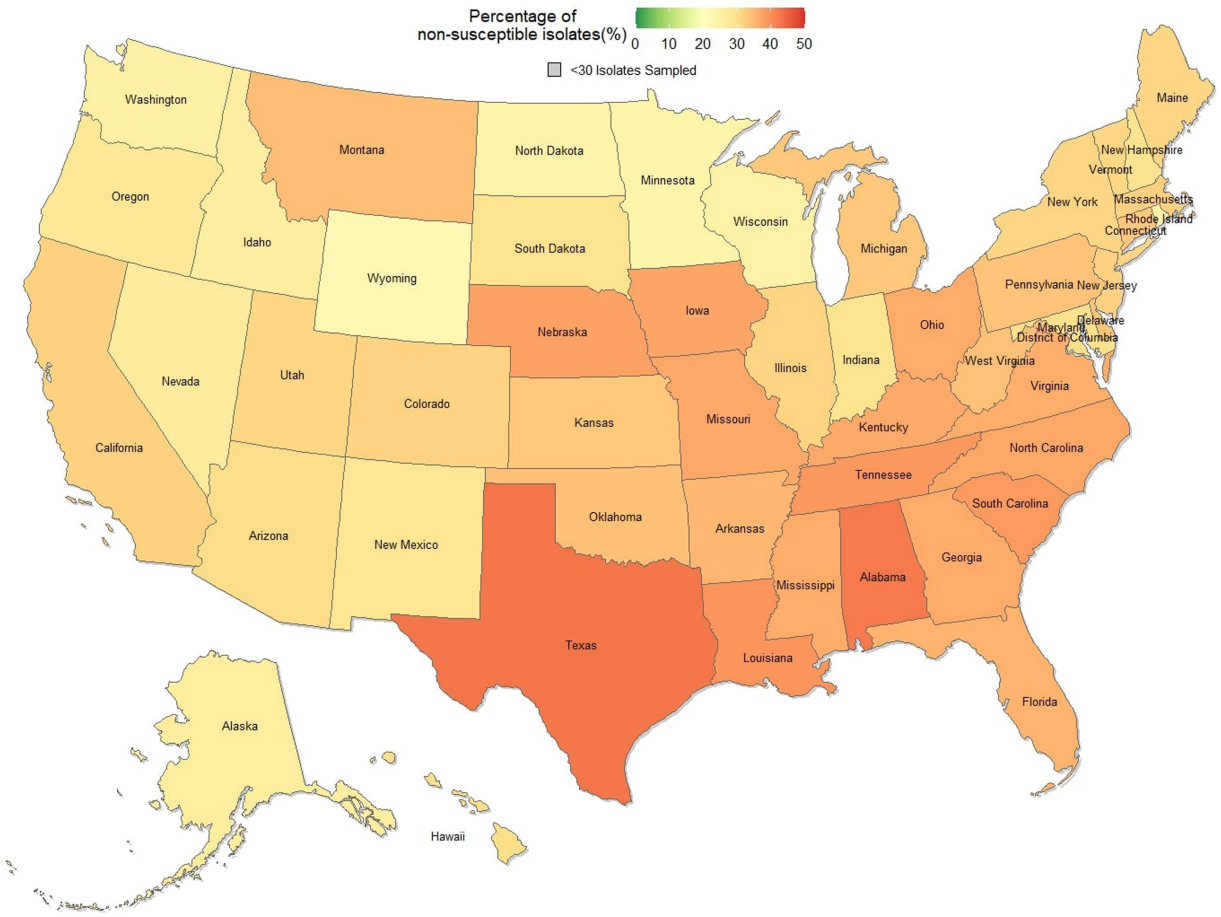

FIGURE 7
Map of the United States, depicting the percentage of *S. pseudintermedius* isolates from cats and dogs found to be resistant to methicillin/oxacillin between 2019 and 2021.

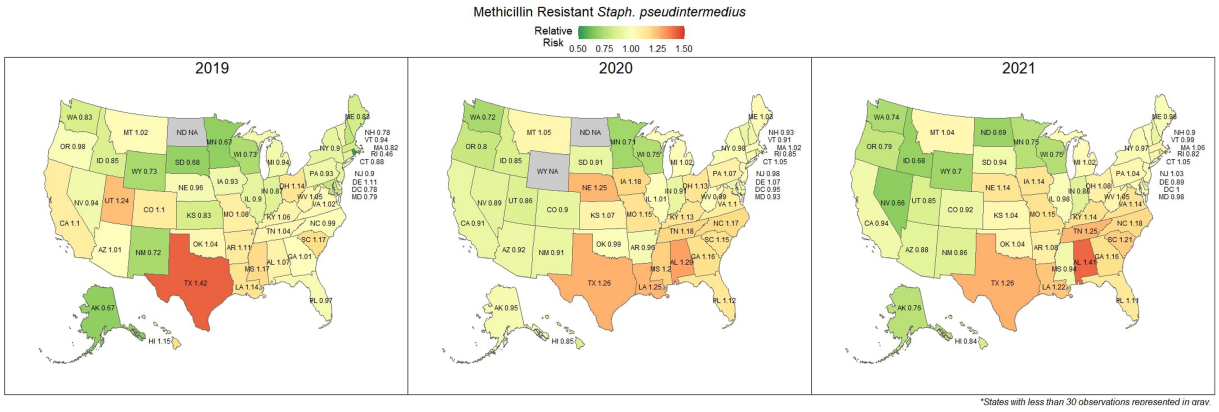

FIGURE 8
Maps of the United States, depicting the relative risks of methicillin/oxacillin resistant *S. pseudintermedius* infections in cats and dogs observed in 2019, 2020, and 2021.

TABLE 2 Comparison of susceptibility test results across drugs of the same class.

	E. coli				*Staphylococcus spp.*			
	Sus.	Res.	Int.	*n*	Sus.	Res.	Int.	*n*
Cephalosporins (III)								
Cefotaxime	88.2%	11.0%	0.9%	322,839	–	–	–	15
Cefovecin	82.1%	16.8%	1.1%	331,080	63.9%	33.1%	3.0%	325,240
Cefpodoxime	83.4%	16.5%	0.1%	331,074	67.0%	32.4%	0.6%	325,067
Ceftazidime	86.0%	12.8%	1.3%	331,073	–	–	–	840
Ceftiofur	85.7%	13.2%	1.1%	315,762	–	–	–	29
Fluoroquinolones								
Ciprofloxacin	90.4%	9.5%	0.1%	331,169	69.8%	29.6%	0.6%	324,939
Enrofloxacin	88.1%	9.5%	2.5%	331,162	65.9%	28.0%	6.0%	325,282
Marbofloxacin	90.4%	9.3%	0.3%	331,168	69.7%	29.6%	0.6%	325,297
Pradofloxacin	–	–	–	41	70.6%	22.4%	7.1%	15,896

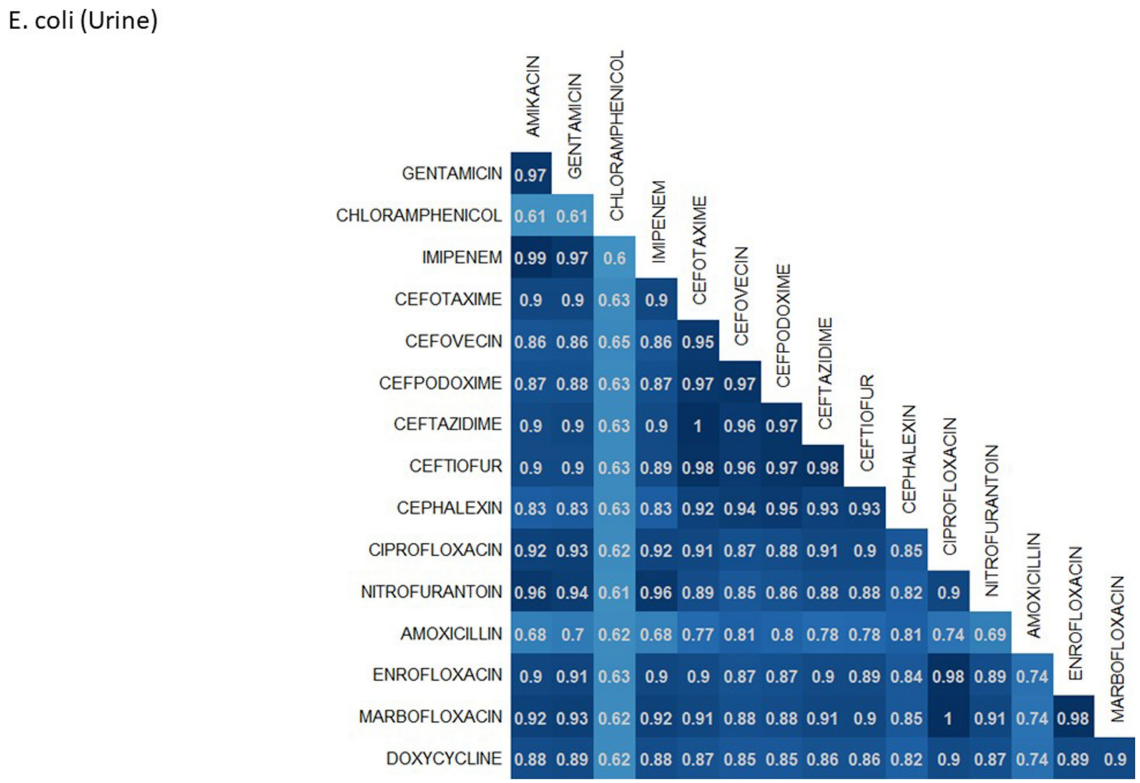

FIGURE 9
Probability matrix for observing the same AST interpretation between pairwise drug combination in *E. coli* isolated from urine samples from cats and dogs.

further into the southern states. A particular pattern of interest was the noticeable switch in relative risk between the east and west coast states for cephalexin resistant *S. pseudintermedius* occurring between 2019 and 2021, for which no explanation can be offered.

E. coli and *S. pseudintermedius* both have the potential to cause zoonotic infections. *E. coli* is a common bacterium found in the gastrointestinal tracts of mammals. This bacterium can be spread through contact with infected fecal matter, causing a range of symptoms, from mild to severe. Several studies have highlighted the zoonotic potential of *E. coli* from companion animal reservoirs, including strains resistant to common antibiotics (30–33). *S. pseudintermedius* is an opportunistic pathogen frequently observed on the skin and mucosa of canines, and to a lesser extent, felines. While often a harmless component of the natural flora, *S. pseudintermedius* may present a zoonotic threat to humans who come into contact with infected companion animals (13, 34). The

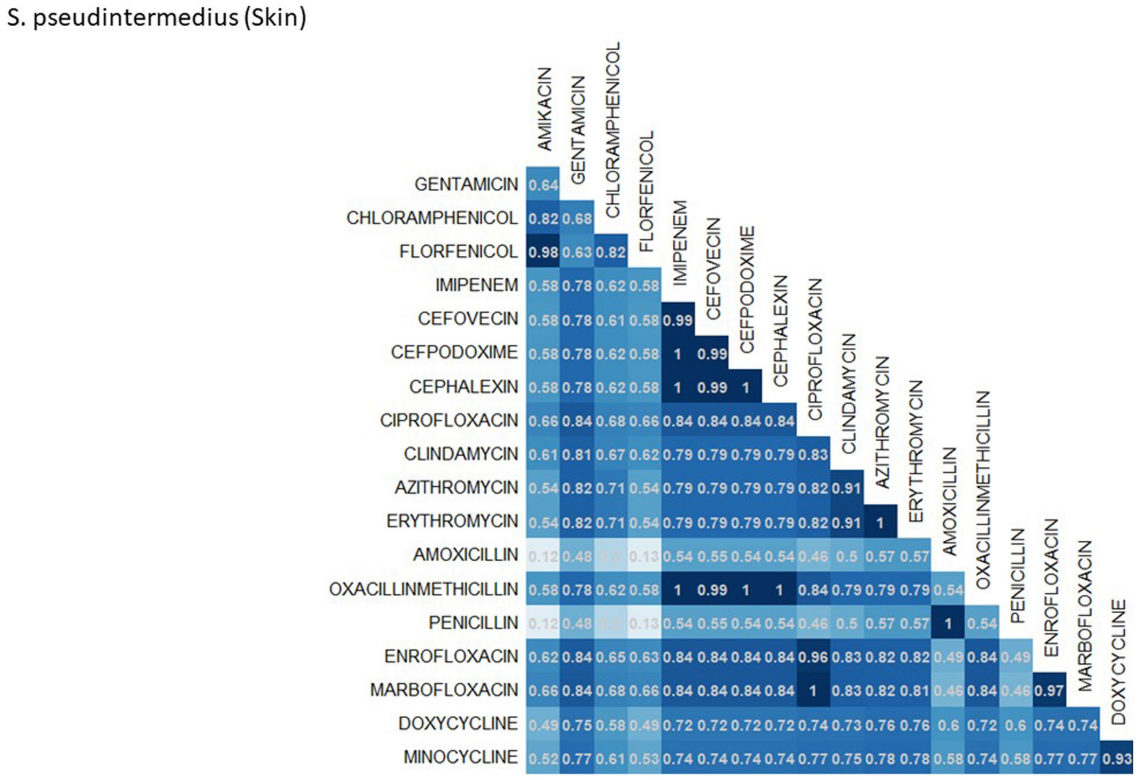

FIGURE 10
Probability matrix for observing the same AST interpretation between pairwise drug combination in *S. pseudintermedius* isolated from skin samples from cats and dogs.

zoonotic potential of both of these bacteria underscore the importance of monitoring for antimicrobial resistance in companion animals which could subsequently spillover into humans and vice versa.

Increasing awareness of multidrug resistant strains of *E. coli* and *S. pseudintermedius* has raised concern for the health of humans and animals (35, 36). Extended spectrum beta lactamases (ESBL) confer resistance to multiple drug classes including third generation cephalosporins, and is becoming more commonly reported in *E. coli* isolates in companion animals (37). Susceptibility to third generation cephalosporins in *E. coli* samples analyzed in this study appears to be high, with a national average of 83.5% of samples showing sensitivity to these drugs. This level was observed consistently across all third-generation cephalosporin drugs tested against *E. coli*. Of notable concern is the discrepancy in resistance between the southern and northern states, as seen in Figure 6. Comparison of relative risk values between these states indicates that a grouping of elevated resistance appears to exist in the southeastern states and should be further assessed. Methicillin resistant *S. pseudintermedius* presents another growing concern for zoonoses and clinical outbreaks in companion animals (38). Looking at all laboratory susceptibility results for *S. pseudintermedius* against methicillin (oxacillin) within the United States between 2019 and 2021 confirms that considerable levels of resistance are being observed in companion animal isolates. On average 32.7% of isolated *S. pseudintermedius* were classified as non-susceptible to methicillin, about ten percentage points lower than the resistance observed to first- generation cephalosporins. Methicillin and first-generation cephalosporins were expected to show greater similarity in their levels of resistance, given that methicillin resistant *S. pseudintermedius* will typically also express resistance to other beta lactams, including cephalexin (39). In the drug agreement matrix (Figure 10), this relationship is observed, as cephalexin and methicillin (oxacillin) showed perfect agreement on all *S. pseudintermedius* samples when tested in parallel (cephalexin and oxacillin both tested on the same isolate). However, when isolates tested against cephalexin, oxacillin or both are included, as is with the mapped state-level resistance, differences were observed. This discrepancy illustrates how this data can be perceived differently based on the inclusion and exclusion criteria and methodologies applied. A three-fold difference in sample size between *S. pseudintermedius* resistance testing against oxacillin and cephalexin may explain this observed discrepancy.

Once more, the resistance observed in a laboratory diagnostics setting is likely to be greater than in the general population, but nevertheless, these findings indicate that MRSP, and to a lesser extent ESBL, are present issues in companion animals of the United States. Higher levels of resistance to first line and subsequent treatments could lead to higher instances of extra-label antimicrobial usage, wherein drugs reserved for human infections are used in companion animals to treat difficult infections (4). Extra-label usage of these drugs may increase the risk of emergence of infections in companion animals resistant to high profile antibiotics in humans such as carbapenems for cephalosporin resistant *E. coli*.

Electronic health records and databases of laboratory antimicrobial susceptibility testing offer the benefit of widespread data coverage as well as consistency in methods to make comparisons over space and time. The results from hundreds of thousands of susceptibility tests, conducted each year, offer interminable information from which numerous hypotheses can be developed and tested at scale, without the need for additional financial resources and patient recruitment. Many epidemiological studies in AMR would be otherwise infeasible, especially those looking to cover a large study area or period of time. This quantity of data points opens the possibility for specialized analysis methods over time and space, which often require sizeable amounts of data but can produce results that cannot be seen at smaller scales. Time series and spatial epidemiology do not answer the question of 'how' but rather highlight the 'where' and 'when' which can be used to allocate resources, direct future research and serve as surveillance tools for early detection of an outbreak.

Further opportunities exist to leverage ongoing laboratory testing into dynamic data visualizations and dashboards for surveillance and decision making. As more samples are processed each day, plots and figures can be continuously updated such that the information is always up to date, as opposed to static plots which can become outdated within a year's time. By involving data science experts, these various information systems at the regional and national level can be made to integrate with one another, allowing for data sets to be combined. Interactive dashboards have become a popular tool in epidemiology to house data visualizations and offer the added functionality of dynamically filtering datasets into specific information pertinent to a given scenario. In this case, the multitude of drug/pathogen/source combinations makes antimicrobial resistance an excellent candidate for this type of tool, one that can be leveraged by researchers for hypothesis generation, as well as clinicians for guiding of antimicrobial treatment decisions. Furthermore, dashboards can be made to automatically receive, process and publish new data with little need for human involvement. The ability to filter down to a specific region, infection site, pathogen, and drug combination could be an invaluable tool, however, could also pose significant risk to antimicrobial stewardship efforts if not thoughtfully produced. Failure to recognize the limitations and biases of AST datasets, and a fundamental understanding of how they may differ from the status of the general population could see veterinarians reaching for higher priority antimicrobials more frequently if first line treatments are represented as uncommonly effective. Clinicians should be involved in the dashboard development process to understand how these tools will be perceived and how the data will be used to make informed decisions.

Although laboratory susceptibility testing presents many opportunities for research into the epidemiology of antimicrobial resistance, several notable data concerns exist and must be assessed. Data such as these contain large amounts of information regarding patient, location and pathogen factors, but two key pieces of information that are not present are the reason why culture specimens were submitted and whether there was prior antimicrobial treatment of that infection (i.e., testing in response to treatment failure). Susceptibility testing is a key part of proper antimicrobial stewardship practices but is not always feasible due to time or cost constraints, or pressure from the client to prescribe treatment quickly. As a result, clinicians may often prescribe a common first line antibiotic, and only submit a sample for testing if that treatment fails, in order to find a suitable replacement. This inherently results in the sampled population of these datasets being representative of more resistant pathogens than in the general population due to sampling bias. However, the extent of this bias is unclear and with limited information regarding why the test was ordered it can be difficult to assess and correct. Further work should seek to understand the motivations of clinicians for both testing and prescribing of antimicrobials, with the aim of understanding the extent of bias within these data sources. Estimates of resistance in the present work should be considered as over-estimations until these biases can be adequately assessed. Additionally, large quantities of data can lead to problems in analysis which can require special consideration and methods to overcome. Simple statistical tests will often find significance when using large datasets due to the exceptionally large statistical power. Therefore, practical significance should always be considered even when statistically significant.

Antimicrobial resistance is a complex health issue, firmly situated in the realm and responsibilities of a One Health framework. Historically, companion animals have been neglected in the AMR discourse, due at least in part to the lack of available population level data to conduct epidemiological studies (15). However, this study has demonstrated the usefulness of deidentified commercial laboratory data to assess epidemiological resistance patterns across a large study area. Using these datasets removes a primary barrier to the inclusion of companion animals into One Health studies and offers a missing piece to the puzzle. In addition, these data can be used as a basis for AMR surveillance in the veterinary community and allow for data-driven decision making in empirical therapy. Numerous in-depth analyses will come from access to such data and can be used to monitor known, emerging and novel resistance concerns.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: privately owned commercial dataset. Requests to access these datasets should be directed to IDEXX Laboratories, Inc.

Ethics statement

Ethical review and approval was not required for the animal study because this study utilized de-identified secondary data. Written informed consent for participation was not obtained from the owners because this work is in compliance with IDEXX terms and conditions which are agreed upon at the time of test ordering, and all personal information was removed.

Author contributions

AP and DS: data extraction and preparation. KS, JW, ZP, and TB: analysis and interpretation of results and draft manuscript preparation. All authors were involved in the study conceptualization and design, reviewed the results, and approved the final version of the manuscript.

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Conflict of interest

AP and DS were employed by IDEXX Laboratories, Inc. TB holds the IDEXX Chair in Emerging Technologies and Preventive Healthcare. KS was supported by the IDEXX Chair.

The remaining 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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Perceptions of antimicrobial stewardship: identifying drivers and barriers across various professions in Canada utilizing a one health approach

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Introduction: As antimicrobial resistance (AMR) represents a substantial threat to the efficacy of available antimicrobial options, it is important to understand how to implement effective and practical mitigation efforts, including antimicrobial stewardship (AMS), across human, animal, and environmental sectors.

Methods: A mixed-methods questionnaire was distributed virtually to attendees of the virtual One Health Antimicrobial Stewardship Conference (March 10–12, 2021) and their professional networks. Respondents (n = 81) were largely from the veterinary (75%) or human (19%) health sectors. Qualitative data were analyzed in NVivo using template analysis whereas quantitative data were analyzed in STATA using Kruskall-Wallis tests. The questionnaire asked respondents about their perceptions of AMS, as well as the perceived barriers and drivers of AMS efforts.

Results: Perceptions of what AMS meant to the respondents personally and their profession as a whole were grouped into 3 main themes: 1) AMS strategies or considerations in antimicrobial prescribing and use; 2) responsibility to maintain health and preserve antimicrobial effectiveness; and 3) reducing antimicrobial use (AMU) as a goal of AMS efforts. Identified AMS barriers had 3 main themes: 1) lack of various prescribing and AMU support mechanisms; 2) shift in prescriber attitudes to drive change; and 3) stronger economic considerations to support shifting prescribing practices. Drivers of AMS had the following themes: 1) leadership to guide change; 2) education to support optimizing AMU; and 3) research to identify best practices and opportunities for action. Across all questions, 2 cross-cutting themes emerged: 1) a One Health understanding of AMS; and 2) blame placed on others for a lack of AMS success.

Conclusion: Overall, sector-specific, but particularly cross-sectoral AMS drivers and barriers were identified, highlighting the importance of a One Health approach in AMR research and mitigation.

KEYWORDS

antimicrobial stewardship, antimicrobial resistance, one health, Canada, drivers and barriers

Introduction

Human, animal, and agricultural crop health rely heavily on effective antimicrobials to treat and prevent microbial infections (1). However, pathogen resistance to antimicrobials threatens the ability to effectively treat infections in humans and animals, where global health and socio-economic impacts are projected to be substantial (1). Antimicrobial use (AMU) is the most important driver of the global increase of antimicrobial resistant (AMR) infections (2, 3). As the same active antimicrobial ingredients are used in products destined for use in humans, animals, and the environment, antimicrobial-bacterial interactions impacting AMR development are complex and multifaceted (4, 5).

The natural environment has a large role in maintaining AMR genes and organisms (5, 6). Environmental reservoirs of AMR pathogens and genes (i.e., in soil and water) represent a source of resistant genetic elements for pathogens of potential concern (7). Therefore, a One Health approach, involving key stakeholders in human, animal, and environmental sectors is required to coordinate efforts toward AMR mitigation (1).

To mitigate AMR, responsible use of antimicrobials is essential. Antimicrobial stewardship (AMS), defined as "multifaceted approaches required to sustain the efficacy of antimicrobials and minimize the emergence of AMR," (8) is an important priority in overall AMR mitigation efforts (5, 9). Successful AMS requires coordinated actions to preserve antimicrobial effectiveness in Canada and beyond and is an important focus of the Pan-Canadian Action Plan on AMR (9). The Pan-Canadian Action Plan recognizes that Canada must take coordinated action in a One Health approach to minimize detrimental impacts of increased resistance to antimicrobials and to preserve their effectiveness (9).

Some AMS programs have been initiated in Canada, including voluntary AMU reduction initiatives, integrated AMU and AMR surveillance programs, prescribing guidelines, resources to support prescribing in a variety of health contexts, and ongoing research to support best practices, antimicrobial alternatives, and diagnostics (9–14). However, according to the most recent Canadian Antimicrobial Resistance Surveillance System Report (14), human infections with AMR pathogens of concern have increased from 2016 to 2020, including community-acquired bloodstream infections with methicillin-resistant *Staphylococcus aureus* (MRSA). In addition, the quantity (measured by weight) of medically important antimicrobials sold for use in all animals in Canada increased by 6.5% from 2019 to 2020 (14). Although reductions of AMU in some production animal industries have been described, when considering treatments based on animal weight (population corrected unit or PCU), the mg/PCU fluctuated over the last decade (14, 15). Considering the One Health implications of AMU and AMR, improving AMS in Canada in all sectors is of utmost importance.

To safeguard antimicrobial efficacy, it is crucial to improve our understanding of how to optimize using available antimicrobials in all sectors (5). Antimicrobial prescribing and use in the human and veterinary sectors are influenced by a multitude of factors including knowledge, previous experiences, and patient/client expectations, as well as broader context and norms of AMU (16–20). Broader influences include, but are not limited to, access to healthcare services, geographical area, socio-economic factors, and time constraints (19, 20). By better understanding various stakeholder perspectives of AMS in general and of ongoing AMS efforts, current initiatives could be adapted, or new initiatives developed that meet identified practical needs and improve uptake and/or impacts.

Specifically, to improve AMS efforts, it is crucial to better understand what drivers and barriers of AMS practices exist across various stakeholders to identify areas for improvement, and to guide AMS conversations and future research questions. There may also be opportunities to harmonize ongoing efforts across sectors and identify current support for a shared goal.

In March 2021, the Alberta Veterinary Medical Association, with support from Alberta Agriculture and Forestry, the AMR – One Health Consortium, and the National Collaborating Centre for Infectious Diseases, hosted a virtual One Health AMS Conference. The virtual environment facilitated a diverse complement of Canadian participants working in the human-animal-environment AMR/AMU/AMS space. To benefit from the wide variety of stakeholders groups and professions included in this event, a mixed-methods questionnaire was developed to identify: (1) perceptions of AMS from a variety of professions in Canada, and (2) drivers and barriers Canadian participants experience in their professions regarding AMS practices.

Materials and methods

The University of Calgary Conjoint Faculties Research Ethics Board approved this study (REB21-0209).

Participant recruitment

This study was conducted with participants of the virtual One Health Antimicrobial Stewardship Conference (March 10–12, 2021) and their professional networks. The conference included >400 attendees from 6 continents, including 26 countries, and spanned the human, animal, and environmental health sectors.

The questionnaire was advertised throughout the conference via email, and an URL and a QR code were made available through the virtual conference portal and followed an informed consent process. After the conference, a reminder email was sent to conference participants and to selected professional networks (Alberta Veterinary Medical Association and the College of Physicians and Surgeons of Alberta). The questionnaire remained open until May 15, 2021.

There were 74 of the 377 Canadian conference participants (21) that completed the questionnaire, resulting in approximately a 20% response rate, whereas 7 additional respondents did not attend the conference but were recruited through their professional networks. Only responses from Canadian participants (93%; 81/87 total participants) were included in the analyses to understand the Canadian context. Questionnaire participants were categorized into sectors (veterinary, $n = 61$; human, $n = 15$; agricultural, $n = 2$; both veterinary and human, $n = 2$, undefined = 1) based on reported profession or reported area of focus for professions that could represent any or more than one sector (i.e., academia).

Questionnaire

A questionnaire was developed using an online survey platform (Qualtrics, Provo, UT, United States) to capture perceptions of AMS,

as well as perceived drivers and barriers of AMS, as they related to the respondents' profession. The questionnaire was developed by the One Health at UCalgary team and piloted internally.

The questionnaire contained 8 Likert scale questions where participants were asked to indicate their level of agreement regarding statements about their personal opinions of AMS, and the perceived opinion within their profession, on a 5-point scale (strongly disagree, disagree, neutral, agree, strongly agree), as well as 2 yes/no questions regarding the perceived existence of AMS drivers and barriers within their profession (see Appendix I). Not all questions were required to be completed for participants to submit their results.

To elucidate perceptions of AMS, participants were also asked the following open-ended questions: "What does antimicrobial stewardship mean to you in your profession?" and "What does antimicrobial stewardship mean to your profession as a whole?" Regarding existing barriers to AMS, participants were asked, "Do you believe there are barriers in antimicrobial stewardship in your profession?" Participants who responded 'yes' were asked the following: "What is preventing antimicrobial stewardship in your profession?"

To understand existing AMS drivers, participants who responded, 'yes' to "Do you believe there is support in place to promote/encourage antimicrobial stewardship in your profession?" were then asked, "What is currently in place that helps promote antimicrobial stewardship in your profession?"

Data analyses

Quantitative analyses were conducted in STATA (Version 15.1, StataCorp LLC, College Station, TX, United States). In addition to inclusion of descriptive statistics, a non-parametric test (Kruskal-Wallis) was used to explore if years of experience or professional sector influenced responses to the Likert scale and to yes/no questions. Years of experience was divided into 2 categories ($\leq$17 and > 17 years, based on the mean years of experience being 17). Statistical significance was accepted when $p \leq 0.05$.

Qualitative data were analyzed using template analysis and a matrix analysis to elucidate differences in AMS perceptions, and AMS drivers and barriers between the sectors. Template analysis provides structure through hierarchical coding, in which sub-themes are classified under main themes (22). This approach was chosen to identify potential cross-sectoral themes and allow for sector-specific components to be coded in the hierarchical framework. Given that using a One Health approach to further understand AMS drivers and barriers in Canada is a novel approach, themes were identified through inductive coding, to enable themes to emerge organically, allowing for flexibility in coding.

In qualitative analysis, quantity of responses under each theme does not necessarily reflect their importance. Rather, the themes that emerge, regardless of how many times they emerge, is of most importance (23). Therefore, commonly mentioned AMS drivers and barriers are important, but so are those mentioned less frequently as they still impact overall AMS success.

Two researchers (KDM and JB) with NVivo (QSR International, Pty Ltd., Version 12) training conducted data coding. Preliminary inductive theme identification was done by independent review of qualitative responses and compared, followed by discussions regarding emerging themes until agreement was reached. Then, main themes and sub-themes were finalized before the first round of coding was conducted independently using NVivo by creating nodes. The second round of coding was conducted by comparing independent coding results and nodes were adapted as required. Any differences among results were discussed to ensure agreement in coding.

A matrix analysis was conducted in Microsoft Excel to organize responses to open-ended questions by sectors to evaluate potential differences in themes between sectors (24). The research team concluded that data saturation (i.e., inductive thematic saturation) was reached at data analysis completion, based on non-emergence of new themes (25).

Results

Participants

A total of 81 Canadians encompassing a variety of professions across the One Health spectrum participated in this study. Participants worked in the following professions/areas and could indicate >1 profession category (Table 1). Participants had a median of 15 years of experience in their profession (range: 1–45 years; mean = 17 years). A total of 74 questionnaire participants attended the virtual One Health Antimicrobial Stewardship Conference, whereas 7 did not attend.

Quantitative data

Participants agreed that AMS was important in AMR mitigation (99%; 79/80 agreed or strongly agreed), whereas there was less agreement regarding whether their profession promotes AMS (80%; 63/79 agreed or strongly agreed), and whether it is viewed as important by their colleagues (76%; 61/80 agreed or strongly agreed) (Table 2).

There was agreement regarding the importance of AMS in livestock and companion animal health for maintaining human health (93%; 74/80 agreed or strongly agreed and 92%; 73/79 agreed or

TABLE 1 Reported professions or areas of focus of participants (participants could indicate <1), and years of experience.

Professions/areas (*N* = 81)	No.	Years of experience Mean (Range)
Veterinary clinician	36	19.9 (2–45)
Academia	31	14.7 (1–44)
Industry	12	15.6 (2–32)
Government	11	10.0 (1–25)
Medical association*	8	28.4 (12–44)
Producer	6	14.7 (2–30)
Producer association	6	17.4 (2–30)
Veterinary technician	3	10.3 (2–15)
Pharmacist	3	30.3 (25–41)
Physician	1	44
Laboratory technician	1	0

*Human or veterinary medical association.

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TABLE 2 Participant responses to Likert scale statements regarding antimicrobial stewardship displayed on a heat map to indicate frequency of responses from least common (white) to most common (red).

Statements (*N*=80)	Strongly disagree	Disagree	Neutral	Agree	Strongly agree
My profession is actively engaged in promoting antimicrobial stewardship[1]	1% (1)	4% (3)	15% (12)	46% (36)	34% (27)
Antimicrobial stewardship is viewed as an important consideration by my colleagues	3% (2)	5% (4)	16% (13)	52% (42)	24% (19)
I have adequate support/resources to ensure antimicrobial stewardship in my work	6% (5)	18% (14)	25% (20)	44% (35)	8% (6)
Antimicrobial stewardship is important in mitigating the threat of antimicrobial resistance	–	–	1% (1)	18% (14)	81% (65)
I believe there is more I could do personally to improve antimicrobial stewardship in my profession	1% (1)	8% (6)	26% (21)	52% (42)	13% (10)
Antimicrobial stewardship in livestock is important for human health	1% (1)	1% (1)	5% (4)	24% (19)	69% (55)
Antimicrobial stewardship in companion animals is important for human health[1]	–	1% (1)	6% (5)	32% (25)	61% (48)
Antimicrobial stewardship in humans is important for livestock health	2% (2)	2% (2)	13% (10)	35% (28)	48% (38)

[1]*N*=79.

strongly agreed, respectively). However, agreement regarding the importance of human AMS for maintaining livestock health was slightly less (83%; 66/80 agreed or strongly agreed). Approximately half (51%; 41/80) of participants felt they had adequate support/resources to ensure AMS in their profession, and 65% (52/80) agreed there was more they could personally do to improve AMS in their profession.

Neither sector nor years of experience influenced participant responses to the majority of Likert scale statements. However, human sector participants tended to be more likely to 'strongly agree' whereas those in the veterinary sector were more likely to 'agree' ($p=0.06$) regarding the statement that their colleagues viewed AMS as important. Veterinarians and veterinary technicians were slightly less positive (68%; 26/38 agreed or strongly agreed) compared to other professions (82%; 35/42 agreed or strongly agreed) regarding their colleagues' views on AMS ($p=0.02$). Further, veterinarians and veterinary technicians had a higher tendency to agree that AMS in livestock is important for human health (97%; 37/38 agreed or strongly agreed) compared to other professions (88%; 37/42 agreed or strongly agreed) ($p=0.052$).

A total of 80% (63/79) of participants believed there were AMS barriers in their profession and 72% (56/78) believed there was AMS support in their profession. Responses by sector are in Table 3. No human sector participant responded 'No' regarding existence of AMS barriers and drivers. However, human sector participants with less experience (≤ 17 years) were more likely to respond "I do not know" regarding existence of AMS barriers compared to their colleagues with more experience (> 17 years) who were confident that AMS barriers existed ($p=0.02$). There were no significant differences between sector responses to yes/no questions.

Qualitative data

The matrix analysis highlighted similarities and differences across the sectors in responses. However, for questions identifying drivers and barriers, due to questionnaire design only asking participants indicating they believed either existed, reduced the number of participants responding to each open-ended question. Therefore, only participants from the veterinary and human health sectors provided responses regarding AMS drivers while one agricultural sector participant also contributed perceived AMS barriers. Therefore, qualitative responses should be considered in primarily the veterinary and human health sectors contexts, and it is indicated where differences emerged between these sectors.

Perceptions of AMS

Participants provided various considerations in AMS strategies and prescribing decision-making, and they shared the overarching goal of AMS as limiting AMU when possible. The following main themes emerged: (1) AMS strategies or considerations in antimicrobial prescribing and use, (2) responsibility to maintain health and preserve antimicrobial effectiveness, and (3) reducing AMU as a goal of AMS efforts. Hierarchy of themes summarizing participant responses that emerged through the inductive coding process are provided in Figure 1.

AMS strategies

The AMS strategies or considerations in antimicrobial prescribing and use theme represents thought processes or considerations reported as part of participant's antimicrobial prescribing or use, or as 1 participant described: "*Striking a balance between required use and perceived need*" (Veterinary clinician, C1N41).

In their responses, participants provided broad descriptions of AMS with either concise or vague language to summarize concepts or various AMS strategies, rather than practical, actionable components (i.e., reducing inappropriate use versus increasing vaccine uptake to reduce need for AMU). Some participants included both conceptual and actionable components in their perceptions of AMS, highlighting evidence-based antimicrobial prescribing.

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TABLE 3 Participant responses ($n = 79$) to questions on the perception of antimicrobial stewardship drivers and barriers within different professions.

	Do you believe there are barriers in improving antimicrobial stewardship in your profession?			Do you believe there is support in place to promote/encourage antimicrobial stewardship in your profession?		
Sector	Yes	I do not know	No	Yes	I do not know	No
Veterinary* ($n = 59$)	49	5	5	42	8	8
Human health ($n = 15$)	11	4	–	11	4	–
Agriculture ($n = 2$)	2	–	–	1	–	1
Human health and veterinary ($n = 2$)	1	1	–	1	1	–
Undefined ($n = 1$)	–	–	1	1	–	–

*$N = 58$ responses for "Do you believe there is support in place to promote/encourage antimicrobial stewardship in your profession?"

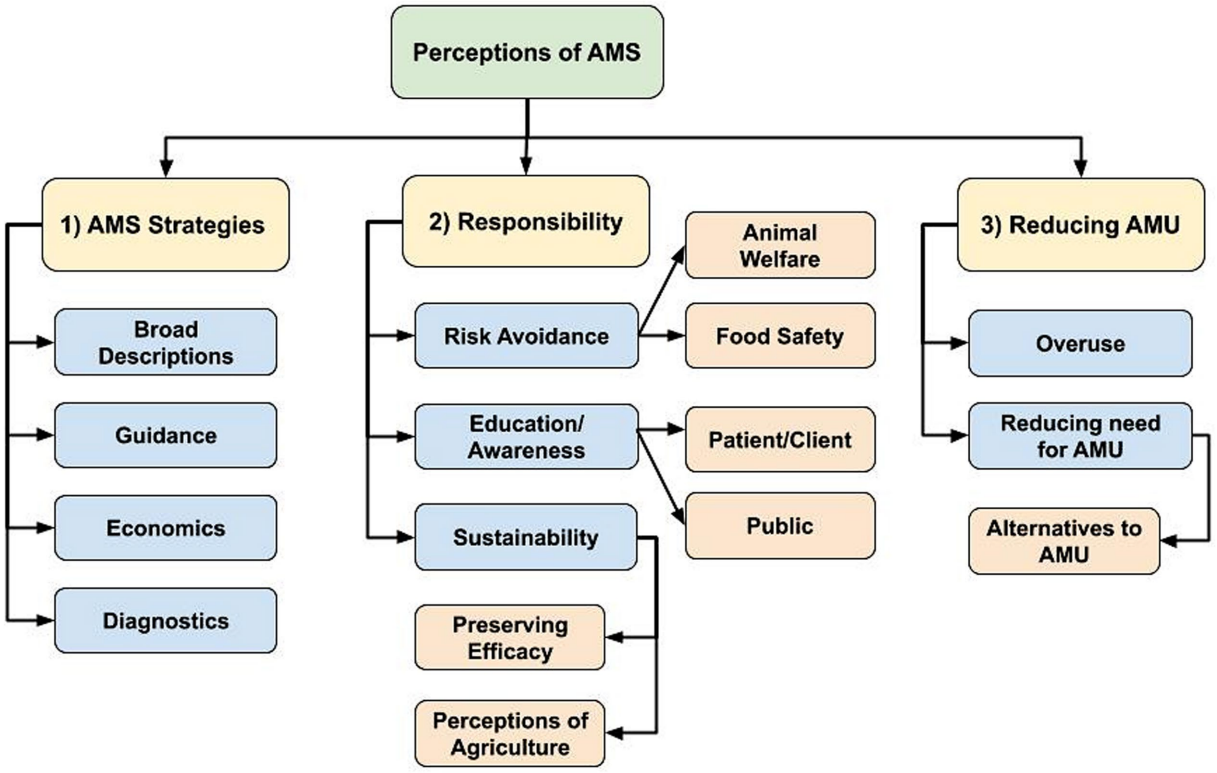

FIGURE 1
Flowchart of identified hierarchical themes across participant responses to the open-ended questions: (1) "What does antimicrobial stewardship (AMS) mean to you in your profession?" ($n = 74$; veterinary sector = 57, human health sector = 15, agricultural sector = 1, both veterinary and human health sector = 1), and (2) "What does antimicrobial stewardship mean to your profession as a whole?" ($n = 73$; one human health sector participant did not respond). (1) AMS strategies or considerations in antimicrobial prescribing and use, (2) Responsibility to maintain health and preserve antimicrobial effectiveness, (3) Reducing antimicrobial use (AMU) as a goal of AMS efforts.

> "[*Stewardship*] *Means a dynamic process of refining strategies to preserve the access to effective antimicrobials to maintain animal health and welfare. The main components of stewardship are* (1) *strategies to ensure proper use of antimicrobials when indicated. This is what we consider veterinary oversight* (*right drug, dose, duration, frequency route*); *and* (2) *The strategies that can be implemented to avoid the use of antimicrobials when possible, such as facilities design, vaccination strategies, genetic selection, handling systems,* etc. *Stewardship initiatives also involve a large component of education and knowledge translation.*" – Veterinary clinician/Medical Association participant (C1N17).

Participants identified guiding factors influencing their AMS strategies, referring to regulations and policies aimed to reduce or control AMU, prescribing guidelines, using antimicrobials according to label instructions, and the importance of a valid patient-prescriber relationship.

Veterinary sector participants mentioned economic considerations influencing AMS strategies as they placed importance

in ensuring both profitability for the producer and food affordability for the consumer.

> *"Responsible and judicious use of antimicrobial products (anti-parasitic products also included) to preserve human, animal and environmental health and welfare, while ensuring the production of safe and affordable food products for human consumption."* – Veterinary clinician (C1N59).

Diagnostics were also mentioned in both the human and veterinary sectors, primarily as the basis for antimicrobial prescribing decision-making. Specifically, bacterial culture and sensitivity testing were described as key components of AMS efforts, supporting evidence-based prescribing. Diagnostics were also mentioned regarding time limitations (i.e., the ability, or lack thereof, to provide a rapid diagnosis to guide antimicrobial choice and limited broad-spectrum AMU), and integral to ongoing AMR surveillance efforts.

Responsibility

It was evident in participant responses that AMS was synonymous with responsibility. This theme represented the context of AMU decisions (i.e., responsible AMU), with regards to personal or moral responsibilities participants placed on themselves regarding individual, day-to-day decisions, plus larger professional and societal duties to optimize AMU.

> *"Responsible use is something the profession is focused on. Realizing we will have to use them but need to put some thought into how we are using them."* – Industry/Human sector participant (C1N56).

The theme of risk avoidance emerged as the prescriber responsibility to maintain health of their patients via antimicrobial prescribing strategies. Food safety and animal welfare were mentioned solely by veterinary and producer participants as important considerations regarding AMU and AMS. Animal welfare was described as a moral responsibility to maintain animal health and welfare in addition to maintaining food system safety and productivity. Veterinarians and producers cited their obligations to the animals under their care, but also to humanity.

> *"Protection of the public. Safeguarding and assuring availability of antimicrobials for future treatment of humans and animals."* – Medical Association/Veterinary sector participant (C1N18).

In the human health sector, risk avoidance was also described as the prescriber's responsibility to maintain health and increase treatment success through AMU. However, risk avoidance also referred to minimizing AMR development by encouraging or facilitating AMU.

> *"In Family Medicine it means using the right antibiotic for the right patients, at the right dose for the right duration, and checking to ensure there are no harmful effects."* – Physician/Academic/Medical Association participant (C1N40).

Education and awareness of AMS and AMR were mentioned as important personal and professional responsibilities. Specifically, antimicrobial prescribers and end-users were considered to have the responsibility to be aware of their actions and potential contributions to AMR. Further, prescribers referred specifically to continuing education (CE) being their responsibility to continually improve prescribing practices, but also that their role was to educate patients/clients and facilitate public awareness and AMS support.

> *"I have a responsibility to follow guidelines regarding appropriate antimicrobial use and educate the public regarding the importance of minimizing drivers of AMR."* – Veterinary clinician/Medical Association participant (C1N45).

> *"To be [a] steward and effectively translate knowledge for public health professionals on AMR."* – Academic/Human sector participant (C1N44).

Participants referred to AMS practices as sustainable use of antimicrobials and overall responsibility to safeguard effective treatment options for future generations. Preservation of antimicrobial efficacy was considered an integral component of AMU sustainability, as well as sustaining human and animal health in general by ensuring future access to antimicrobials.

> *"The responsibility to use antimicrobials in a prudent and sustainable manner in order to preserve the use for the future and reduce current and future harm."* – Veterinary clinician/Academic/Producer/Medical Association participant (C1N12).

Finally, the veterinary sector described their responsibility to maintain positive perceptions of agriculture, as consumer safety and animal welfare contribute to maintain a social license to use antimicrobials in animal production and to production system sustainability.

Reducing AMU

The goal of reducing AMU was described as both reducing antimicrobial overuse and the need for AMU. Reducing the need for antimicrobials encompassed both prevention (i.e., limiting the need for AMU through various infection prevention and health improvement initiatives) and alternative treatment options to antimicrobials.

> *"Reduction of inappropriate exposure of antibiotics to help maintain antibiotic effectiveness for infection treatment."* – Government/Human sector participant (C1N38).

> *"We are looking for alternative ways to improve animal health without the use of antimicrobials."* – Academic/Veterinary sector participant (C1N47).

Many participants viewed their role in AMS not just as 'appropriate prescribing,' but also as educators and facilitators promoting stewardship and preventing unnecessary AMU.

> "*To me, antimicrobial stewardship means reducing inappropriate use of antimicrobials. It means educating those who prescribe and use antimicrobials. It means questioning prescriptions when there is insufficient evidence to determine appropriateness."* – Pharmacist/Human sector participant (C1N60).

Barriers to AMS

Participants described a vast array of existing AMS barriers that are both sector and profession-specific but were also experienced across sectors. Regarding AMS barriers, there was emergence of 3 main themes: (1) lack of prescribing and AMU support mechanisms, (2) a required shift in prescriber attitudes to drive change, and (3) a need for stronger economic considerations to support shifting prescribing practices (Figure 2).

AMU support mechanisms

The AMS barrier regarding the described lack of support to optimize antimicrobial prescribing and AMU practices had various sub-themes, including the lack of access to certain antimicrobials, potentially limiting appropriate antimicrobial selection. Juxtaposition of the desire to reduce AMU coupled with required antimicrobial access for treatment was present, as well as the need for diagnostics to inform prescribing decisions. Participants expressed that widespread availability of effective antimicrobial alternatives is currently lacking.

Participants indicated they did not have access to enough educational opportunities to support their own personal knowledge as well as identified limited collective understand through limited research/knowledge in certain areas (i.e., to support development and implementation of best practices to optimize AMU and limit the need for AMU) to support required AMS education and resources.

The general lack of consequences if prescribers failed to meet AMS guidelines was identified as a barrier, or according to 1 participant, the "*intangible consequences of antimicrobial misuse*" (Pharmacist/Human sector participant C1N60). Participants described a general lack of antimicrobial prescribing oversight, and a lack of agreement regarding AMS in general, including clearly defined best practices. Participants stated if decisions were made regarding best practices, they were not communicated to enable everyone to clearly understand what is required.

> *"Family practice training programs do not have strong enough emphasis and monitoring of what we do."* – Physician/Academic/Medical Association participant (C1N40)

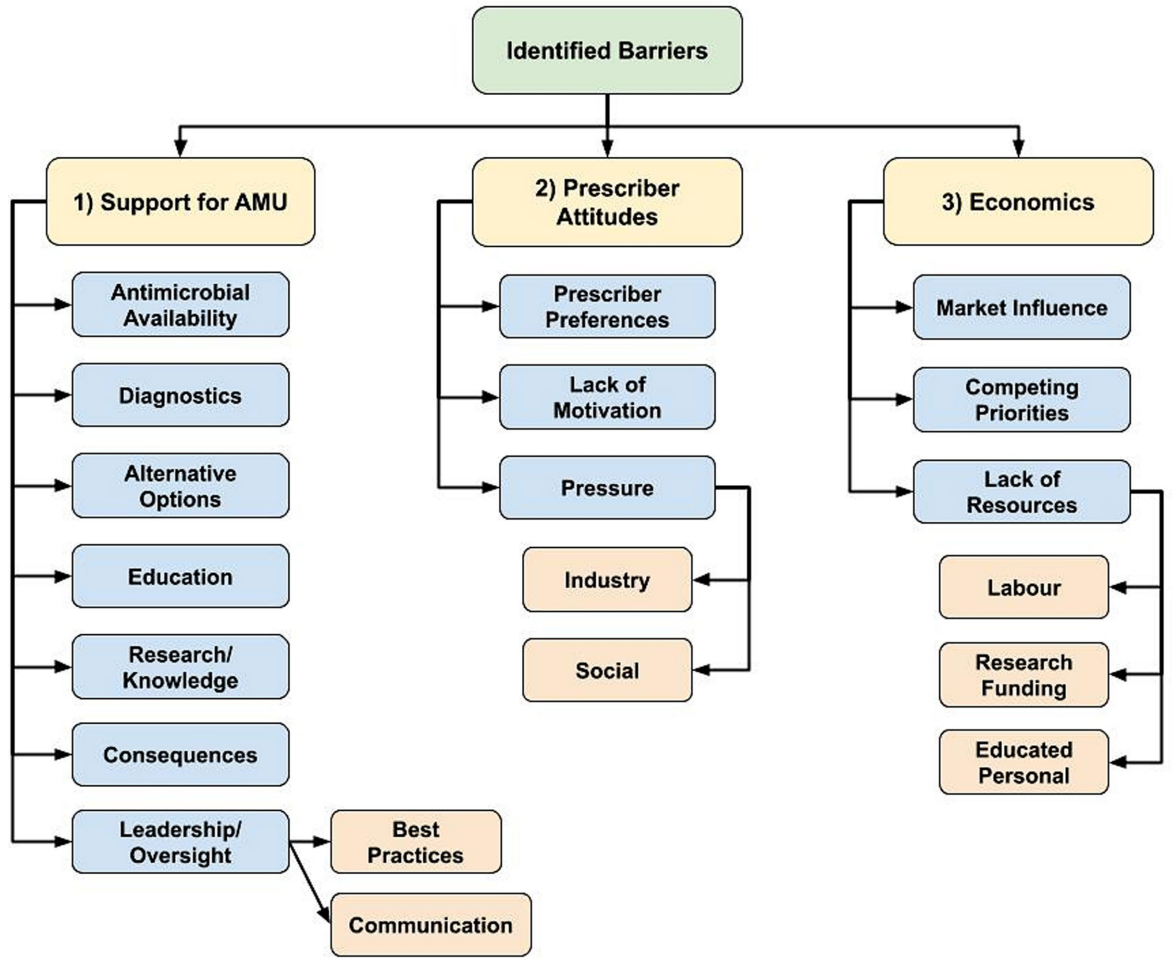

FIGURE 2
Flowchart of identified hierarchical themes across participant responses to the open-ended questions about antimicrobial stewardship barriers: "What is preventing antimicrobial stewardship in your profession?" (n = 59; veterinary sector = 47, human health sector = 11, agricultural sector = 1) posed to participants who responded, 'yes' to "Do you believe there are barriers in antimicrobial stewardship in your profession?" (1) Lack of various prescribing and antimicrobial use (AMU) support mechanisms, (2) Shift in prescriber attitudes to drive change, (3) Stronger economic considerations to support shifting prescribing practices.

> *"There are no simple steps or actions producers or farmers can start implementing tomorrow or this evening. As a vet tech and producer, I know I should change my farming practices, but even I don't know the first step."* – Veterinary technician/Producer participant (C1N32)

The lack of communication and collaboration between stakeholders at various healthcare system levels was identified as a barrier, contributing to a limited shared understanding of responsibilities.

> *"There is support from groups, governments, industry,* etc.*, but there is a lack of consensus and collaboration between these in their messaging and impact."* – Veterinary clinician/Academic/Industry/Producer Organization participant (C1N35).

> *"Lack of awareness and understanding between professions. It seems like at times we are ahead and at times others are. We should all be on the same page, consistently."* – Government/Human sector participant (C1N58).

> *"Engaging more stakeholders especially environmental health professionals."* – Academic/Human sector participant (C1N44).

Prescriber attitudes

Prescriber attitudes and an overall lack of motivation to change behaviors were described as maintaining current levels of antimicrobial prescribing by supporting "*old habits or protocols for treatment*" (Veterinary clinical/Medical Association participant C1N45), or there being a "*lack of an overall driving force to get this done*" (Academic/Veterinary sector participant C1N28).

> *"Many field practitioners may agree that antimicrobial stewardship is important but at the end of the day they do not change their behaviors due to preference, finances, external pressures,* etc.*"* – Veterinary clinician/Industry participant (C1N9).

Participants working as antimicrobial prescribers described the pressures they experience, and realities of working in healthcare. Social pressures were described as the public expectation that a healthcare visit automatically results in a prescription for them or their animal. Participants felt that a prescription has become part of the social contract of healthcare for the visit to feel like it had value. Industry pressure including intensive animal production, the pharmaceutical industry, and lack of antimicrobial alternatives were all considered to contribute to AMU.

> *"Client pressure and outcome motivators put pressure on [the] profession."* – Industry/Human sector participant (C1N56).

Economics

Other AMS barriers were economic in nature. This theme was primarily mentioned by the veterinary sector. Market influence, or "*economics of agricultural production*" (Veterinary clinical/Medical Association participant C1N33) was highlighted as being an important barrier, which included small profit margins and a lack of economic incentives to improve AMS.

Competing priorities were also described, such as the inherent inconsistency in private veterinary clinics between selling antimicrobials for profit and supporting AMS. Producers described being in a similarly difficult position, needing to balance fear of potential disease and profit impacts when withholding antimicrobials or limiting prophylactic AMU, and supporting AMS. One participant stated that "*Current production systems do not allow for/encourage adoption of alternative practices that may decrease/better target antimicrobial use*" (Veterinary clinician/Producer/Industry participant C1N61).

Economic limitations experienced by veterinary clients were also mentioned as limiting prescribing abilities to support AMS practices, including the cost-prohibitive nature of using diagnostic tools to optimize AMU or aid in antimicrobial selection.

> *"Because of financial constraints (of clients) veterinarians often do not have culture and sensitivity results on which to base therapeutic choices, and scheduling recheck examinations can be more difficult in veterinary than in human patients."* – Veterinary clinician/Academic participant (C1N13).

Further, the lack of cost-effective antimicrobial alternatives, and limited financial capacity to make structural changes to reduce infection rates to limit the need for antimicrobials (i.e., improvements in biosecurity or animal husbandry) were identified as important barriers. Labor constraints (i.e., time and capacity of employees) and a lack of educated personnel were also identified as reducing the ability to make improvements that support AMS.

> *"A lack of cost-effective, efficient methods to address reduced use of antimicrobials is also a barrier."* – Veterinary clinician/Government participant (C1N14).

Drivers of AMS

Regarding drivers of AMS, there was emergence of 3 main themes: (1) leadership to guide change, (2) education to support optimizing AMU, and (3) research to identify best practices and opportunities for action (Figure 3). Whereas lack of progress in these themes presented as AMS barriers, their mention as drivers was accompanied by some examples of existing programs or support. However, the overarching theme in response to the question about existing AMS support was the general lack of support participants felt to improve AMS practices.

Examples of existing AMS leadership and guidance included regulations and professional prescribing guidelines. Additionally, examples of easily accessible educational opportunities and resources to support and drive AMS practices were provided by participants, including CE opportunities, conferences and websites. "*Guidelines and CE from professional organizations*" (Veterinary Clinician/Medical Association participant C1N10) were described as important sources of information for prescribers.

Research to better understand AMU best practices and to identify areas for AMU reduction were described as AMS drivers.

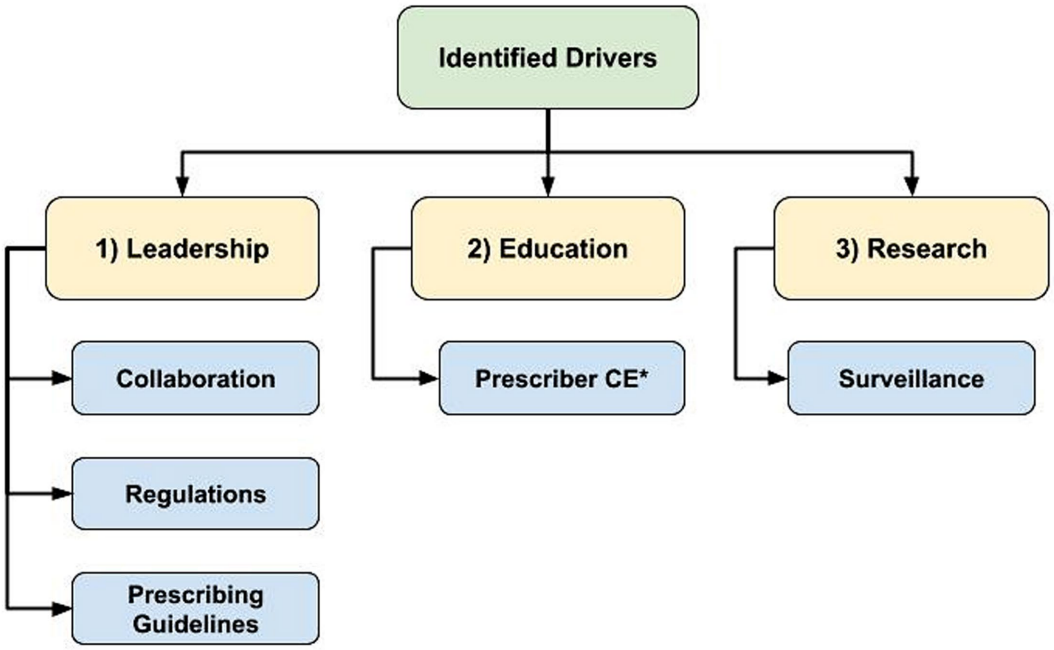

FIGURE 3
Flowchart of identified hierarchical themes across participant responses to the open-ended questions regarding antimicrobial stewardship drivers: "What is currently in place that helps promote antimicrobial stewardship in your profession?" (n = 47; veterinary sector = 37, human health sector = 10) asked to participants who responded 'yes' to "Do you believe there is support in place to promote/encourage antimicrobial stewardship in your profession?" (1) Leadership to guide change, (2) Education to support optimizing antimicrobial use, (3) Research to identify best practices and opportunities for action, *CE = Continuing Education.

Active AMU/AMR surveillance programs identifying usage trends and changes in prevalence of important resistant pathogens were also deemed important. Although participants provided some examples of existing AMS drivers, many responses indicated that there was not enough AMS support.

> *"There are many programs and information available to help guide decision making, lots of CE efforts. However, the lack of specific information in some instances (ex. limited guidelines in equine practice) and lack of awareness among clinicians are remaining barriers."*– Veterinary Clinician (C1N12).
>
> *"Written strategies exist or are being developed. More work needs to happen to promote the concepts within them."*– Veterinary Clinician/Medical Association participant (C1N33).

Cross-cutting themes

Across participant responses to multiple questions, there was emergence of 2 cross-cutting themes: (1) a One Health understanding of AMS, and (2) blame placed on others for the lack of AMS success. Although the transdisciplinary nature of AMR was acknowledged in responses, that also translated to blame being placed on others, including other sectors.

One health

Whereas questions centered around how participants perceived AMS, as well as related drivers and barriers of AMS, the One Health concept was pervasive in responses. Some descriptions of AMS included 2 sectors (primarily human and animal health), whereas others included human, animal, and environmental sectors, or specifically the term 'One Health.'

> *"Responsible and judicious use of antimicrobial products to preserve human, animal and environmental health and welfare."* – Veterinary clinician (C1N59).
>
> *"Practicing and educating prudent use of antimicrobials since health of all forms of life is inter-related."* – Academic/Human sector participant (C1N43).

The One Health theme was a pervasive response to the question "Who should take responsibility in promoting antimicrobial stewardship?" (Figure 4). Although participants indicated they believed there should be a top-down approach (i.e., government-led AMS support), they also described that everyone needs to be involved, because "*It's One World, One Health*" (Academic/Human sector participant C1N43).

> "*I think that anyone with knowledge/expertise in antimicrobial resistance should promote antimicrobial stewardship.*" – Academic/Human sector participant (C1N3)
>
> *"Everyone has a role in antimicrobial stewardship. The lead for stewardship programs should be multidisciplinary and include health system leadership."* – Pharmacist/Human sector participant (C1N21)

FIGURE 4
Word cloud of the most common responses (n = 67 participants) to the question "Who should take responsibility in promoting antimicrobial stewardship?"

Blame

Another cross-cutting theme that emerged was blame. Participants placed blame for the lack of current AMS success on others within their profession, as well as on other sectors. Existing industry structures and overall cultural norms were also blamed for the lack of AMS success. Some participants (8%; 6/80) did not agree that their colleagues viewed AMS as important (Table 2), or claimed others had a "*lack of awareness and regard for the issue*" (Government/Agriculture sector participant C1N50).

> *"We all have a part to play in stewardship, but not all may be putting it as a priority in the profession."* – Academic/Veterinary sector participant (C1N46)

Additionally, blame was placed on patients and clients by prescribers for pressuring them for antimicrobial prescriptions, limiting their ability to maintain AMS practices. Prescribers also described receiving blame from patients or clients if treatments were unsuccessful.

There was also blame placed on prioritization of human health over health of other species.

> *"It is not just about safeguarding certain antimicrobials for human use - need to consider impact on [the] rest of [the] species on [the] planet too."* – Veterinary clinician/Academic/Industry/Government participant (C1N72)

Although a perceived lack of AMS support emerged as a barrier across sectors, it also emerged in response to questions regarding existing support. Participants stated that they did not have enough support in AMS activities, and that more support was required for meaningful progress.

> *"More needs to be offered at the level of producers and general public."* – Veterinary clinician (C1N22)

In addition to the perceived lack of AMS support being described in qualitative responses, it was also evident in the Likert scale responses where ~25% of participants claimed that they did not have adequate AMS support (Table 2). Participants expressed that overall, *"We have some support. But not enough."* (Producer/Producer Organization participant C1N15).

Discussion

This study described the presence of a 'status quo' of antimicrobial prescribing and use in the Canadian context, maintained by described barriers to improving AMS. Participants felt personal responsibility in AMS, but ~25% of participants did not feel they had adequate support to improve AMS. A total 80% of participants believed AMS barriers existed in their profession; the few participants indicating AMS barriers did not exist in their profession were from the veterinary sector or was a participant with an undefined profession. Human sector participants suggested that the certainty regarding the existence of AMS barriers ("I do not know" versus "Yes") increased with time spent in the profession, which may reflect barriers individuals experience over time as they consider AMS in their profession.

Skepticism regarding AMU in animals and the subsequent impact on AMR in humans is common in the veterinary sector (26–28). However, our results indicated there was overall agreement among participants that AMS in livestock was important for humans, especially among veterinarians and veterinary technicians, but less so regarding the converse. Regardless, transmission of human AMR pathogens to animals has been identified, as well as broader impacts of human AMU and its contributions to environmental contamination and AMR are important (5, 7).

This perceived species hierarchy in AMR is reiterated in descriptions of AMS practices in livestock, where the main goal is maintaining safe food systems for humans, instead of solely focusing on animal health. In that regard, a focus on animal health to maintain human health reflects the global focus of public health where livestock AMS efforts are required to preserve antimicrobials important for human health (13, 29), but there are not necessarily policies in place to ensure the reverse. However, animal health and welfare should be prioritized, highlighted by veterinary sector participants as a moral responsibility of care and reflected in the literature (27, 28, 30).

Many participants viewed the concept of AMS to be synonymous with responsibility in terms of contributing to the AMS education of others and food safety, and most importantly, preservation of antimicrobial efficacy. However, there is an inherent contradiction in combining aims of preventing and managing bacterial infections in a risk-averse manner through antimicrobial treatment and preservation of antimicrobial efficacy for future infections (e.g., increased antimicrobial prophylaxis for COVID-19 patients during the pandemic) (31, 32). The desire to use antimicrobials to avoid potential negative clinical outcomes through practices such as prophylaxis, or 'future discounting' was described by UK producers and veterinarians working in a variety of livestock industries (27). Motivation to limit AMU existed but is contradicted by concern for potential animal welfare or production impacts when antimicrobials are withheld (27). Furthermore, human hospital personnel described antimicrobial prescribing being influenced by professional liability (33).

As described by participants, as a prescriber or antimicrobial user, it is difficult to assign specific negative impacts to AMU in general, or

providing preventative or prophylactic antimicrobials, when impacts of increasing AMR are not immediate or clearly visible. This concern for harmful immediate impacts by withholding antimicrobials, coupled with the intangible consequences of antimicrobial misuse and the pressure put on prescribers, could contribute to an overall lack of motivation to change prescribing practices.

Unfortunately, Canadian investment in AMR has been stagnant in the past decade (34). Participants noted that it may be necessary to rethink our current health and agricultural systems to further support AMS. One important consideration is the access and cost of timely diagnostics in both the human and animal contexts, as well as the cost of other infection prevention and control measures to limit the need for antimicrobials. Further, in the current private veterinary clinic model, there is financial reliance on selling products to clients, including antimicrobials. It will be a challenge to shift our current health and agricultural systems to further support AMS from an economic perspective, although that could increase sustainability.

Specifics of how to alter each production system or healthcare context to support AMS would need to be investigated further in collaboration with stakeholders within each context. This should also include economic considerations that support sustainability of production industries as well as contribute to shared goals with pharmaceutical industries to support prolonging efficacy of antimicrobial products. As described by participants, substantial health system changes may be required to further entrench AMS priorities, including reconsidering animal production systems to improve biosecurity and reduce the need for AMU while remaining profitable, or improving market support for novel antimicrobial research and development (35).

Lack of overall leadership and stakeholder collaboration was described as an AMS barrier. Collaboration between leaders in AMS and key stakeholders at all levels in healthcare is required to effectively drive AMS efforts (36). However, prescribers' resistance to other healthcare provider recommendations and a lack of continuity of care were identified as AMS barriers by acute care hospital personnel in Nova Scotia (33). Although a top-down approach of AMS governance was identified by participants as required for AMS improvement, they also described a need for collaboration at all levels of antimicrobial prescribers and end-users. Co-development of AMS goals and protocols within healthcare teams can serve to involve all relevant healthcare team members in the AMS discussion (33). Opportunity to influence change is a characteristic of successful implementation (37).

Increased public involvement and communication could also help limit the public pressure on prescribers for prescriptions, and limit overall antimicrobial misuse. To support efforts in AMS stakeholder communication, education in AMS efforts is integral to success; however, it should not be the sole focus of an intervention (38).

Understanding the role of the environment in the AMR ecosystem has been identified as an important knowledge gap (5). Participants identified the environmental component in AMS collaboration as lacking and that more engagement should be sought. Expanded communication and collaboration across sectors are required with a One Health approach, and essential to overall AMR mitigation success.

The cross-cutting theme of blame highlights the occasional divisions within and between sectors. Blame can contribute to feelings of apathy regarding stewardship efforts (27). 'Other blaming' is a common theme that emerges in AMS research, where some stakeholders feel reluctance of other stakeholders to act renders their efforts to be pointless (27, 39, 40). Antimicrobial prescribers or users could feel that their AMS efforts are being negated or diluted by the overprescribing or use of others (27). To combat feelings of apathy toward stewardship, increased transparency and accountability, or collaboration in general could help make people feel like they are working towards the same goal (27).

Finally, when asked who should take responsibility for promoting AMS, the most common response was that everyone shares responsibility in AMS efforts. The One Health concept was evident in responses, with responsibility being placed on antimicrobial prescribers and users in all sectors, as well as government, industry, professional associations, researchers, diagnosticians, and educators. Although the One Health understanding of AMR was clear and responsibility was placed on all sectors, so was blame for lack of success. However, if the barrier of poor communication and collaboration can be improved to develop a national and global sense of collective AMS responsibility, meaningful progress may be made.

The goal of the qualitative analysis was to describe responses from Canadian participants. Study design limitations included small sample sizes for the human healthcare ($n = 15$) and environmental sectors ($n = 2$) regarding quantitative responses which could have led to the overrepresentation of veterinary sector specific responses. Limitations also include potential bias for increased awareness, or belief of AMS importance and emergence of the One Health theme due to participating in an AMS-focused One Health conference ($n = 74$). Further, the virtual nature of the questionnaire limited the ability to explore participants perspectives deeper, compared to an open-ended study design conducted in person. Regardless, the study design allowed for convenient questionnaire distribution and could contribute to critical discussion of AMS barriers due to assured anonymity. Despite study limitations, results presented highlighted various themes and key components of AMS in a One Health framework to address AMR in Canada.

Conclusion

Participants across sectors viewed AMS in Canada as important, with personal and professional responsibility and sustainability of AMU representing major themes across sectors. The described sense of responsibility can be capitalized on to prioritize AMS as "*a target to be achieved*" (Veterinary clinician/Academic participant C1N34) across sectors and professions in pursuit of a shared goal. Participants clearly identified the importance of One Health in AMS, placed blame on others and acknowledged there was more that they could do personally to improve AMS in their profession. Both sector-specific and cross-sectoral AMS drivers and barriers were identified, highlighting the diverse needs of required AMS improvements in Canada.

Data availability statement

The datasets presented in this article are not readily available because the informed consent process referred specifically to the use of collected data for the preformed analysis and manuscript preparation. Requests to access the datasets should be directed to KM, kayley.mccubbin@ucalgary.ca.

Ethics statement

The studies involving human participants were reviewed and approved by the University of Calgary Conjoint Faculties

Research Ethics Board (REB21-0209). The patients/participants provided their written informed consent to participate in this study.

Author contributions

KM prepared the manuscript with supervision from HB, RA, and SL. KM led the questionnaire development and study implementation, with contributions from EJ, RA, SL, SO, and HB. JB contributed to the data analysis, whereas A-MS and JI contributed significantly to the manuscript preparation. All authors contributed to manuscript revision, as well as read, 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.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1222149/full#supplementary-material

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Case report: A case of brucellosis misdiagnosed as coronavirus disease 2019/influenza in China

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Brucellosis is an important zoonosis and a multisystem disease. The signs and symptoms of brucellosis are not specific. In the clinical, brucellosis is often ignored and misdiagnosed. We report a case of brucellosis who was misdiagnosed as coronavirus disease 2019 (COVID-19)/influenza and received delayed treatment during strict COVID-19 control. The neglect of other diseases due to COVID-19 and empirical diagnosis and treatment by medical staff are part of the reasons for misdiagnosis. Otherwise, the normal erythrocyte sedimentation rate (ESR), increased white blood cell count (WBC), and increased neutrophil count (NEUT) of this patient was also a cause of misdiagnosis, which is an important reminder for diagnosis. For patients with the unknown origin of fever and other symptoms related to brucellosis, especially those from endemic areas of brucellosis, brucellosis screening is a priority item, and grassroots doctors should be vigilant and standardize the diagnosis and treatment based on epidemiology history, clinical manifestation, and laboratory tests according to the diagnostic criteria of brucellosis.

KEYWORDS

brucellosis, coronavirus disease 2019, misdiagnosis, erythrocyte sedimentation rate, febrile diseases

Introduction

Brucellosis is an important worldwide zoonosis. Human infections are primarily acquired through contact with infected animals and their secretions (1, 2). Human-to-human transmission takes place through blood transfusion, bone marrow transplantation, and mother-to-fetus transmission (3). The signs and symptoms of brucellosis are not specific, fever, sweat, fatigue, and joint ache are the most common manifestation of human brucellosis, these are similar to influenza, severe colds, coronavirus disease 2019 (COVID-19), malaria, and other infectious diseases, so clinical diagnosis is difficult in place of lack of health facility and specific and rapid diagnostic methods (4, 5). An epidemiological survey based on 2060 cases collected from brucellosis clinics in China showed that 57.62% of patients were misdiagnosed or suspected of having other diseases with similar clinical symptoms (6). Other report indicate that brucellosis is easily misdiagnosed as a variety of other infections and noninfectious diseases (7). In clinical set up, diagnosis of brucellosis is made based on history, clinical manifestation, and laboratory tests including culture, serological tests, and nucleic acid amplification assays. Besides, the

hematological parameters including biochemical examination and blood routine examination are commonly observed in the diagnosis of brucellosis (8–11).

Because of the COVID-19 pandemic, China has taken strict measures to control patients with fever. It is crucial for the prognosis to identify non-COVID-19 infections in patients with fever as early as possible. If patients with brucellosis fail to receive timely and standardized treatment, the probability of cure will be greatly reduced (12). The disease is more likely to progress to a chronically incapacitating disease with severe complications, which affect patients' working ability and life quality (13). Here, we report a *Brucella* case misdiagnosed as COVID-19/influenza. Overly strict management of COVID-19, neglect of normal erythrocyte sedimentation rate (ESR), and increased white blood cell count (WBC) and neutrophil count (NEUT) of the patient caused the misdiagnosis of brucellosis, which is a warning for the diagnosis and treatment of brucellosis amidst the pandemic.

Case presentation

On October 5, 2022, a 61 years-old male patient was presented to the local hospital following a one-week history of fever [38.5°C(101.3°F)], systemic muscle and joint pain, and burning sensation in the skin. The patient came from a remote county in the Altun Mountain region of Gansu province in western China, which is a brucellosis endemic area. A survey on the epidemic of brucellosis in this area showed that the brucella seroprevalence in livestock was 4.2%, and that of human population was 1.2% (14). The hospital treated him as a suspected case of COVID-19. Between October 8 and October 11, he tested negative for COVID-19 on 4 consecutive days, based on the quantitative polymerase-chain-reaction (qPCR) test at the local center for disease control and prevention. Subsequently, he self-administered a herbal medicine Ganmaoling granule which consists of eight main ingredients: Ye Ju Hua (Flos *Chrysanthemi indici*), Jin Zhan Yin Pan (*Bidens biternata* Merr. et Sherff), Gang Mei (Radix *Ilex asprella*), San Cha Ku (Radix *Evodia lepta*), caffeine, acetaminophen, chlorpheniramine maleate, and menthol oil. Although the fever was temporarily alleviated, other symptoms worsened. On October 18, the patient went to the local hospital for treatment, and the COVID qPCR was again negative, but his white blood cell count and other blood indicators increased, indicating serious infection (Table 1). The patient was diagnosed with influenza with bacterial infection without any pathogenic or serological examination, and was clinically treated with metamizole sodium, intramuscular injection of penicillin, and oral sulfanilamide. Thereafter, the symptoms were slightly relieved. However, for nearly a month from October 25 onwards, he started experiencing sleep hyperhidrosis and had a fever (98.6–100.4°F) from 4 to 5 AM every day. After oral metamizole sodium, the temperature returned to normal, but the patient continued to feel ill. After further inquiry, it was understood that the patient raised cattle and sheep infected with *Brucella melitensis*. In addition, a sheep had a miscarriage, which was confirmed to be infected with *B. melitensis*, and the patient handled the aborted animal without personal protection (15, 16). No one else contact with the infected animals and aborted foetus, there was no confirmed case of brucellosis in his family members and neighbors.

On November 25 the Rose-Bengal plate test (RBPT) of the patient's serum was positive. The *Brucella* serum antibodies titer was tested by Wright agglutination test, and the result was 480 I.U/mL (17). Data from routine surveillance for brucellosis showed that the patient's serum antibody test for brucellosis was negative on 28 July. But the blood culture and PCR tests for *Brucella* were negative on 25 November. As local patients are generally unwilling to undergo bone marrow puncture, and the hospital is located in a remote area of China with limited medical level, doctors lack experience in bone marrow puncture, so bone marrow culture is not performed. To avoid the *Yersinia enterocolitica* serotype O:9 which may lead to cross-reactivity in serology, the slide agglutination tests were performed. The results of sera collected from patients at different times were all negative, which ruled out the possibility of *Y. enterocolitica* O:9 infection (18). Although the blood indicators at this time tended to be normal compared with those obtained a month before (Table 1), the patient still experienced muscle and joint pain, and was finally diagnosed with brucellosis infection case which defined as a patient with a history of epidemiologic exposure and associated clinical manifestations of brucellosis, and the test result of Wright agglutination test ≥60 I.U/mL. On November 27, the patient received specific treatment for brucellosis: rifampicin 0.6 g (qd), doxycycline 0.1 g (bid), and silibinin meglumine 150 mg (tid). Rifampicin and doxycycline are both hepatotoxic, while silibinin meglumine has the effect of protecting the liver, so the above three drugs are used simultaneously for treatment. After 1 week of treatment, his condition improved significantly. After 2 weeks of treatment, the Wright agglutination test result had reduced to 240 I.U/mL. After 35 days of treatment, that result was 120 I.U/mL, the symptoms completely disappeared, and the treatment was stopped (Figure 1).

Discussion

To control the spread of the pandemic, COVID-19 has been tightly managed in China until December 2022. In clinical practice, patients with fever were treated as suspected cases of COVID-19. After excluding COVID-19, they were admitted to hospitals for routine treatment, leading to misdiagnosis of some diseases or delayed treatment, such as the pneumonic plague case in Tibet, China, in September 2022 (19). Plague infection wasn't considered in this case, as there was no animal surveillance of plague and no human cases historically in the area. Further, COVID-19 was prevalent in Xigaze, and the hospital only conducted multiple COVID-19 examinations, resulting in delayed treatment and eventual death.

The delayed treatment of the brucellosis case we reported was also caused by misdiagnosis. COVID-19 infection case was defined as a person who meets the clinical manifestations of COVID-19 and the epidemiological history of COVID-19, and tests positive for COVID-19 nucleic acid or antibodies. However, doctors are worried about the spread of the epidemic due to missed diagnosis of COVID-19, so they give priority to treating the patients with fever as COVID-19 infection case and perform multiple tests on the patient without considering the screening of other diseases. Because of the misdiagnosis as COVID-19 and influenza with bacterial infection, the treatment was delayed by nearly 1 month. The diagnosis of influenza with bacterial infection was made empirically. The patient was a herdsman with an obvious epidemiological history. However, because

TABLE 1 Important indicators of the patient's clinical examination.

Indicator	2022.10.18	2022.11.25	2022.11.26	Reference range	Unit
WBC	18.03*	8.66	—	4–10	$\times10^9$/L
NEUT#	13.17*	5.11	—	2–7	$\times10^9$/L
LYMPH#	3.46	3.16	—	0.8–4	$\times10^9$/L
MONO#	1.39*	0.30	—	0.12–1.2	$\times10^9$/L
EO#	0.00*	0.08	—	0.02–0.5	$\times10^9$/L
BASO#	0.01	0.01	—	0–0.1	$\times10^9$/L
IG#	0.21*	0.05*	—	0	$\times10^9$/L
NEUT%	73*	59	—	50–70	%
LYMPH%	19.2*	36.5	—	20–40	%
MONO%	7.7	3.5	—	3–10	%
EO%	0.0*	0.9	—	0.5–5	%
BASO%	0.1	0.1	—	0–1	%
IG%	1.2*	0.5*	—	0	%
RBC	4.57	5.42	—	3.5–5.5	$\times10^{12}$/L
HGB	158	175*	—	120–170	g/L
RDW-CV	12.4	13.0	—	11–16	%
RDW-SD	42.8	43.7	—	35–56	fL
PLT	202	131	—	100–300	$\times10^9$/L
MPV	11.9	11.3	—	6.5–12	fL
PDW	16	16.7	—	15–17	
CRP	57.8*	37.5*	—	0–10	mg/L
ESR	6	—	4	0–15	mm/h
hsCRP	>5*	—	—	0–3	mg/L
Anti-CCP	48.3*	—	—	0–45	U/mL

*Abnormal indicators.
—Indicates that testing was not done.
Indicator interpretation: WBC, white blood cell count; NEUT#, neutrophil count; LYMPH#, lymphocyte count; MONO#, monocyte count; EO#, eosinophil count; BASO#, basophil count; IG#, immature granulocyte count; NEUT%, neutrophil percentage; LYMPH%, lymphocyte percentage; MONO%, monocyte percentage; EO%, eosinophil percentage; BASO%, basophil percentage; IG%, immature granulocyte percentage; RBC, red blood cell count; HGB, haemoglobin; RDW-CV, coefficient variation of red cell distribution width, RDW-SD, standard deviation in red cell distribution width; PLT, platelet count; MPV, mean platelet volume; PDW, platelet distribution width; CRP, c-reactive protein; ESR, erythrocyte sedimentation rate; hsCRP, hypersensitive c-reactive protein; anti-CCP, anti-cyclic peptide containing citrulline.

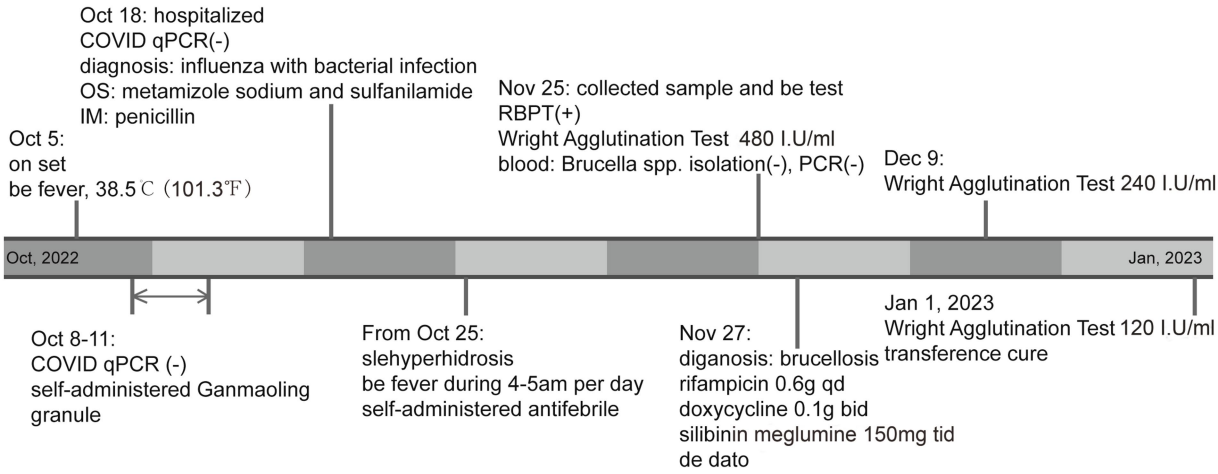

FIGURE 1
Patient symptoms, examinations, and treatment process.

of the lack of basic training in the diagnosis and treatment of infectious diseases, the hospital did not consider infectious diseases and did not query the epidemiological history, leading to misdiagnosis and delayed treatment. The misdiagnosed case of brucellosis is the only case we have collected so far. We found many brucellosis patients in the area, but other patients will voluntarily state the history of brucellosis exposure during the visit, and the doctor will make a differential diagnosis of brucellosis. The patient lives in a region where animal brucellosis is relatively severe. Clinicians should consider brucellosis in patients with unexplained fever, based on their epidemiological history. Empirical treatment with penicillin and sulfanilamide failed to achieve optimal results. Penicillin is ineffective against *Brucella* (20). Sulfanilamide, as a chemical drug, has a certain antibacterial effect on *Brucella*, but it cannot be effectively treated, leading to continued disease development (21).

The hematological parameters including biochemical examination and blood routine examination are commonly observed in the diagnosis of brucellosis. Our patient's ESR was normal, and the patient has increased WBC and NEUT (Table 1), which could also be a reason for misdiagnosis and an important point to remember for future clinical diagnosis of brucellosis. As the highest titer of IgG antibody is produced at around 20 days, it can be seen from the infection process of this patient that his highest Wright agglutination test result was at least 960 I.U/mL. The failure to isolate the pathogen and the negative PCR test in this patient were closely related to the misdiagnosis, and the opportunity for pathogenic diagnosis was lost because of misdiagnosis. Therefore, after excluding other microbial infections, clinicians should make a comprehensive judgment based on epidemiological history, clinical symptoms, and laboratory examinations; carry out immunological examination; and confirm the diagnosis and start treatment for suspected brucellosis cases as soon as possible to improve the treatment effect and avoid acute brucellosis turning into chronic brucellosis.

Currently, COVID-19 and influenza are still prevalent. COVID-19, influenza, and brucellosis have similar clinical symptoms. These three diseases are easy to be misdiagnosed. Other countries have also reported cases of brucellosis misdiagnosed as COVID-19 (22, 23). Those cases also presented symptoms of fever, fatigue and arthralgia and the case 2 reported by Salman et al. was also with a normal ESR (Table 2). Similar to our case, the misdiagnosis or delayed treatment of these two cases were related to the failure to inquire about the epidemic contact history in time. Therefore, in brucellosis endemic areas, the contact history of brucellosis should be confirmed as soon as possible for patients with fever. The possibility of brucellosis should be considered for patients with epidemiology history of contact with infected animals or ingestion of infected meat or unpasteurized dairy products. In addition, the spleen examination of these two cases was abnormal, which also suggested that liver and spleen pathological examination was necessary for the patients with fever to assist in differential diagnosis, and equally important, blood culture should be carried out in time before drug intervention. During the epidemic period of COVID-19 and influenza, patients with fever take antibiotics empirically, which affects the isolation of pathogenic bacteria. The ESR results of the twice tests in this case were normal. Other studies have also reported that patients with brucellosis have normal ESR. Whether this is related to the medication taken by the patients needs further study. This phenomenon suggests that we should pay attention to the influence of drugs on hematological parameters in clinical diagnosis to prevent misdiagnosis due to empirical diagnosis. For patients with the unknown origin of fever and other symptoms related to brucellosis, especially those from endemic areas of brucellosis, brucellosis screening is a priority item, and grassroots doctors should be vigilant and standardize the diagnosis and treatment based on epidemiology history, clinical manifestation, and laboratory tests according to the diagnostic criteria of brucellosis. In addition, complete serological monitoring was performed from the acute phase to recovery for our case. Changes in serologic titers in this patient demonstrate the importance of timely and specific treatment of brucellosis. It was also convenient to grasp the condition for timely adjustment of treatment. This suggests that patients with brucellosis should continue to follow up with the same physician to prevent delay in diagnosis and treatment.

TABLE 2 Symptoms and laboratory examination results of case of brucellosis misdiagnosed as coronavirus disease 2019.

	Our case	Case 1	Case 2	Reference range	Unit
Symptoms					
Fever	Yes	Yes	Yes	—	—
Fatigue	Yes	Yes	Yes	—	—
Arthralgia	Yes	Yes	Yes	—	—
Sweat	Yes	No	No	—	—
Laboratory examination					
WBC	18.03*/8.66	4.5	4.8	4–10	$\times10^9$/L
HGB	158/175*	124	142	120–170	g/L
NEUT#	13.17*/5.11	—	1.6*	2–7	$\times10^9$/L
LYMPH#	3.46/3.16	—	2.9	0.8–4	$\times10^9$/L
PLT	202/131	89*	263	100–300	$\times10^9$/L
CRP	57.8*/37.5*	66.54*	63.95*	0–10	mg/L
ESR	6/4	—	10	0–15	mm/h

Case number: Case 1: reported by Kucuk and Gorgun. Case 2: reported by Salman et al.
*Abnormal indicators.
—Indicates that testing was not done.
Indicator interpretation: WBC, white blood cell count; HGB, haemoglobin; NEUT#, neutrophil count; LYMPH#, lymphocyte count; PLT, platelet count; CRP, c-reactive protein; ESR, erythrocyte sedimentation rate.

Conclusion

We report a misdiagnosed case of brucellosis. During strict COVID-19 control, some diseases were misdiagnosed or received delayed treatment. The normal ESR, increased WBC, and increased NEUT was also a cause of misdiagnosis in this case, which is an important reminder for diagnosis. For patients with the unknown origin of fever and other symptoms related to brucellosis, especially those from endemic areas of brucellosis, brucellosis screening is a priority item, and grassroots doctors should be vigilant and standardize the diagnosis and treatment based on epidemiology history, clinical manifestation, and laboratory tests according to the diagnostic criteria of brucellosis. A better understanding of the clinical significance of hematological parameters and timely improvement of the level of pathogen detection can facilitate early diagnosis and

prevent misdiagnosis of brucellosis. Brucellosis patients should be educated to visit medical specialists rather than paramedics and continue follow-up with the same physician to prevent delay in diagnosis, or unnecessary or wrong treatment, with complication of the unmanaged disease.

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 studies involving human participants were reviewed and approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention of the Chinese Center for Disease Control and Prevention. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

XW contributed to the conception, design of the work, and supervised the work. SQ, RD, and QD performed the experiments. XZ, AB, and XL provided data of patient. SQ, DL, HJ, and MY performed the analysis and interpretation of the data. SQ, DL, and RD drafted the manuscript. XW and XZ reviewed and critically revised the manuscript. 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.

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Publisher's note

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Zoonotic tuberculosis in a high bovine tuberculosis burden area of Ethiopia

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Background: Tuberculosis (TB) is a major cause of ill health and one of the leading causes of death worldwide, caused by species of the *Mycobacterium tuberculosis* complex (MTBC), with *Mycobacterium tuberculosis* being the dominant pathogen in humans and *Mycobacterium bovis* in cattle. Zoonotic transmission of TB (zTB) to humans is frequent particularly where TB prevalence is high in cattle. In this study, we explored the prevalence of zTB in central Ethiopia, an area highly affected by bovine TB (bTB) in cattle.

Method: A convenient sample of 385 patients with pulmonary tuberculosis (PTB, $N = 287$) and tuberculous lymphadenitis (TBLN, $N = 98$) were included in this cross-sectional study in central Ethiopia. Sputum and fine needle aspirate (FNA) samples were obtained from patients with PTB and TBLN, respectively, and cultures were performed using BACTEC™ MGIT™ 960. All culture positive samples were subjected to quantitative PCR (qPCR) assays, targeting IS*1081*, RD9 and RD4 genomic regions for detection of MTBC, *M. tuberculosis* and *M. bovis*, respectively.

Results: Two hundred and fifty-five out of 385 sampled patients were culture positive and all were isolates identified as MTBC by being positive for the IS*1081* assay. Among them, 249 (97.6%) samples had also a positive RD9 result (intact RD9 locus) and were consequently classified as *M. tuberculosis*. The remaining six (2.4%) isolates were RD4 deficient and thereby classified as *M. bovis*. Five out of these six *M. bovis* strains originated from PTB patients whereas one was isolated from a TBLN patient. Occupational risk and the widespread consumption of raw animal products were identified as potential sources of *M. bovis* infection in humans, and the isolation of *M. bovis* from PTB patients suggests the possibility of human-to-human transmission, particularly in patients with no known contact history with animals.

Conclusion: The detected proportion of culture positive cases of 2.4% being *M. bovis* from this region was higher zTB rate than previously reported for the general population of Ethiopia. Patients with *M. bovis* infection are more likely to get less efficient TB treatment because *M. bovis* is inherently resistant to pyrazinamide. MTBC species identification should be performed where *M. bovis* is common in cattle, especially in patients who have a history of recurrence or treatment failure.

KEYWORDS

Mycobacterium bovis, *Mycobacterium tuberculosis*, zoonosis, cattle, central Ethiopia

1. Introduction

Tuberculosis (TB) is among the most significant human infectious diseases worldwide, especially impacting low- and middle-income countries. An estimated 10.6 million new cases and 1.6 million deaths were attributed to TB in 2021 (1). Although the vast majority of TB cases in humans are caused by *Mycobacterium tuberculosis sensu stricto*, other highly related subspecies of the *Mycobacterium tuberculosis* complex (MTBC), such as *Mycobacterium africanum* and *Mycobacterium bovis* can also cause TB in humans. In fact, all species within the MTBC share over 99.9% identity at the genome level (2). Despite this high similarity however, there appears to be host-adaptation among the different MTBC species (3), with *M. bovis* being mainly associated with TB in cattle, also known as bovine TB (bTB).

It has been estimated that 1.4% of all human TB cases in the world, and 2.8% of all cases in the African population, are attributed to *M. bovis* (4, 5). However, the global picture of human TB caused by *M. bovis* is largely incomplete because of reliance on laboratory techniques that are insufficient for accurate differentiation between *M. bovis* and *M. tuberculosis*, including direct smear microscopy, GeneXpert, or culturing of mycobacteria without species-level identification (6, 7). Moreover, human TB caused by *M. bovis* is clinically, radiographically, and pathologically indistinguishable from TB caused by *M. tuberculosis* (8, 9). Hence, the exact contribution of *M. bovis* to the global epidemiology of human TB is possibly underestimated because of underdiagnosis and underreporting, particularly in developing countries where bTB is endemic in cattle and likely not controlled for.

Zoonotic tuberculosis (zTB) has previously been defined as human infection with *M. bovis* (4). More recently, other subspecies of the MTBC have also been identified in cattle and the definition of zTB has been challenged (10). However, for the purpose of this Ethiopian study, we refer to zTB as "TB in humans caused by *M. bovis*" and bTB as "TB in cattle caused by *M. bovis*". Transmission of zTB to humans occurs most frequently through inhalation of aerosol droplets from infected animals, or through ingestion of untreated dairy products carrying *M. bovis* (7). Human-to-human transmission is considered significantly less common (11, 12).

Ethiopia is a highly agrarian society, with over 70% of its nearly 120 million people engaged in agriculture. With an estimated 65 million cattle, its livestock sector has the largest national cattle herd in Africa and the sixth largest in the world (13). Approximately 98% of these cattle are of the local zebu breeds reared extensively by rural smallholders or pastoralists, while the remaining 2% are dairy cattle of exotic breeds—or crosses with the local zebus—that are mostly accommodated in intensive husbandry settings around urban centers (13). Extensive epidemiological studies from the last decades suggest that the prevalence of bTB in cattle in rural settings across Ethiopia is relatively low, with rates of 5–10% (14, 15). However, the intensive dairy sector has been more affected. Several studies in the well-established dairy belt around Addis Ababa in central Ethiopia have reported an average bTB animal prevalence of between 25 and 30%, while the herd prevalence in certain parts has reached 50–60% bTB (16–18). Ethiopia has not yet implemented a bTB intervention program. Only sporadic tuberculin testing and subsequent slaughter of infected cattle have been performed in selected herds but at small scale. Also, a basic post-mortem examination program at slaughterhouses has been introduced. Previous attempts to estimate the prevalence of *M. bovis* in the human population in Ethiopia [which has a TB incidence rate of 143/100,000 population (1)] have suggested a zTB rate of below 1% (19, 20). Most of the sites explored in these studies have however been in regions of the country where the level of bTB in cattle has been relatively low, while studies focusing on zTB in the central parts of the country, where the bTB rate is very high, have been limited. Therefore, we set out to investigate the prevalence of zTB in the central region of Ethiopia to understand whether the very high bTB prevalence in cattle is reflected in the human population. We also discuss the zoonotic impact from the perspective of exposure to cattle and the behavior of raw milk and meat consumption.

2. Materials and methods

2.1. Study design and setting

A multi-center health facility-based cross-sectional study was conducted in central Ethiopia from October 2019 to March 2021. Three hospitals (Adama, ALERT and Bishoftu) and five health centers (Adama, Bishoftu, Sebeta, Holeta, and Sendafa) located in Addis Ababa city and the surrounding zone of Oromiya region were selected to recruit study participants. The high prevalence of bTB in cattle in central Ethiopia was the major reason for this selection. Health centers typically serve 50,000–60,000 people, whereas Bishoftu and Adama hospitals each serve approximately 1.2–1.5 million. An illustrative representation of the study areas is depicted in Figure 1.

2.2. Study population

Patients clinically or microbiologically diagnosed with pulmonary TB (PTB) or TB lymphadenitis (TBLN) were enrolled consecutively upon informed consent. PTB cases were enrolled at all selected study sites whereas TBLN patients were enrolled in hospitals where fine-needle aspirate (FNA) cytology examination by a pathologist was available. Extrapulmonary TB (EPTB) patients other than those with TBLN were excluded from the study.

2.3. Data collection and sampling of clinical specimens

Clinical and demographic information was collected from enrolled patients using a structured questionnaire. Morning sputum

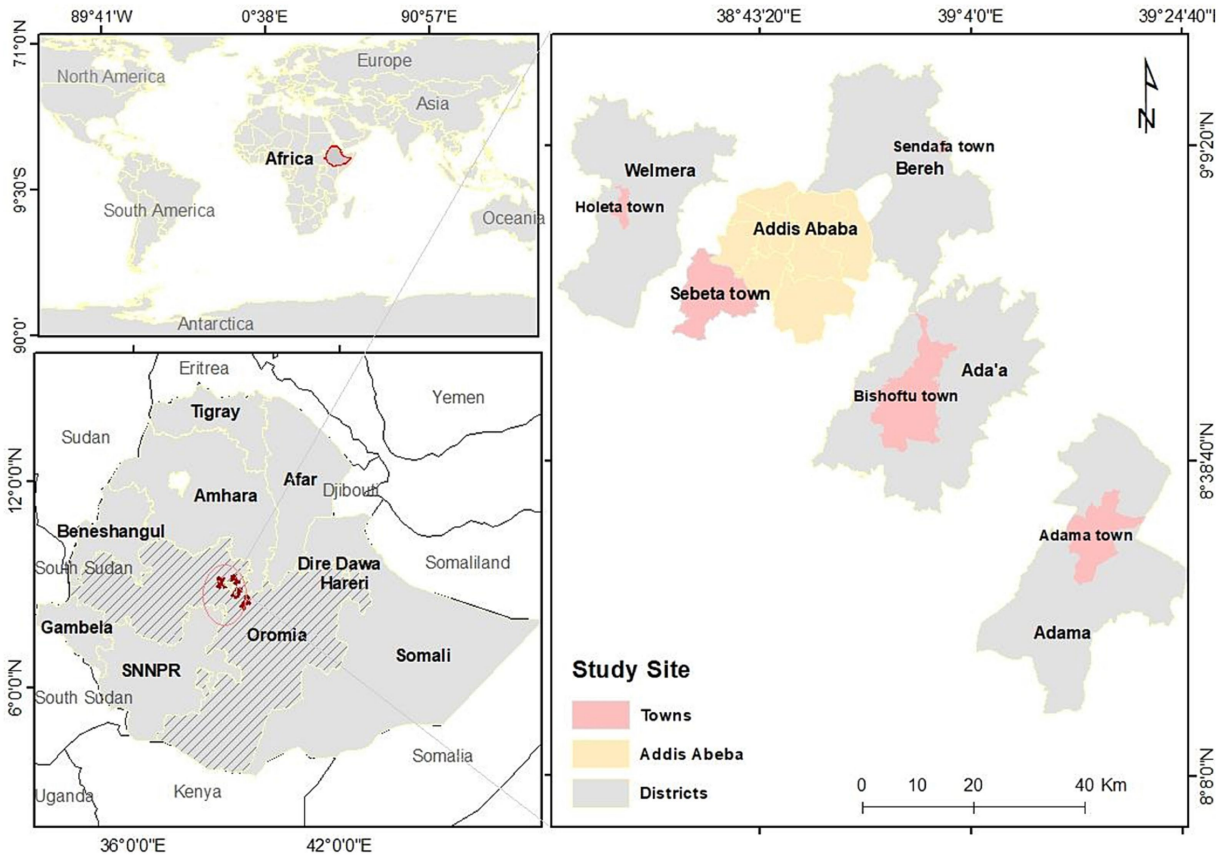

FIGURE 1
Mapping of seven health centers in Addis Ababa and five surrounding towns in central Ethiopia where patients were recruited into this study.

and FNA specimens were collected before the initiation of anti-TB treatment from PTB and TBLN patients, respectively. FNA specimens were collected aseptically by experienced pathologists from enlarged cervical lymph nodes with a 21-gage needle attached to a 10 mL syringe and transferred into cryo-tubes containing 1 mL phosphate buffer saline (PBS) pH 7.2, while sputum specimens were collected in sterile 50 mL plastic tubes. Both sample types were kept at −20°C at the study sites until they were transported on ice (up to +4°C) to the Armauer Hansen Research Institute (AHRI) in Addis Ababa for sample processing and culturing of mycobacteria.

2.4. Culturing of mycobacteria

Mycobacterial culturing was performed on sputum and FNA samples following the procedure indicated in the Mycobacteriology Laboratory Manual (21) using BACTEC™ MGIT™ 960 Mycobacterial detection system. In brief, samples were decontaminated by the standard N-acetyl-L-cysteine and sodium hydroxide (NALC/NaOH) method with a final NaOH concentration of 1%. An equal volume of standard NALC/NaOH solution was added to the specimen and incubated for 15 min. After neutralization with PBS and 15 min centrifugation at 3,000 × g, the sediment was re-suspended in 1 mL of sterile PBS. A volume of 0.5 mL of each re-suspended sample was inoculated into a MGIT liquid medium tube. Inoculated MGIT tubes were placed directly into the MGIT 960 instrument for incubation for up to 48 days or until detection of growth. Heat-killed cells were prepared, by taking 500 µL broth from culture-positive MGIT tubes for incubation at 90°C for 20 min, and used for subsequent molecular identification. In parallel, decontaminated sample volumes remaining after MGIT inoculation were used for Ziehl-Neelsen staining and smear microscopy, and for culture-negative samples, if enough volume remained, genomic DNA (gDNA) was extracted using QIAamp® DNA mini kit (Qiagen) following the manufacturer's protocol and eluted in a volume of 50 µL, and then stored at −20°C until further analysis. A flow chart of the sample collection, processing, and molecular typing is provided in Figure 2.

2.5. Identification of mycobacterial genomic DNA

Quantitative PCR (qPCR) was performed on heat-killed bacterial suspensions or extracted gDNA in the case of culture-negative samples. IS*1081*, RD9, and RD4 were used as target genomic regions for identification of MTBC, *M. tuberculosis*, and *M. bovis*, respectively. IS*1081* is an insertion sequence specific for all mycobacterial species of the MTBC and has been shown to appear as six copies in each genome (22). The IS*1081* assay is expected to be more sensitive than the RD9 and RD4 assays since it is a multiple copy gene (22). Therefore, the qPCR assay with

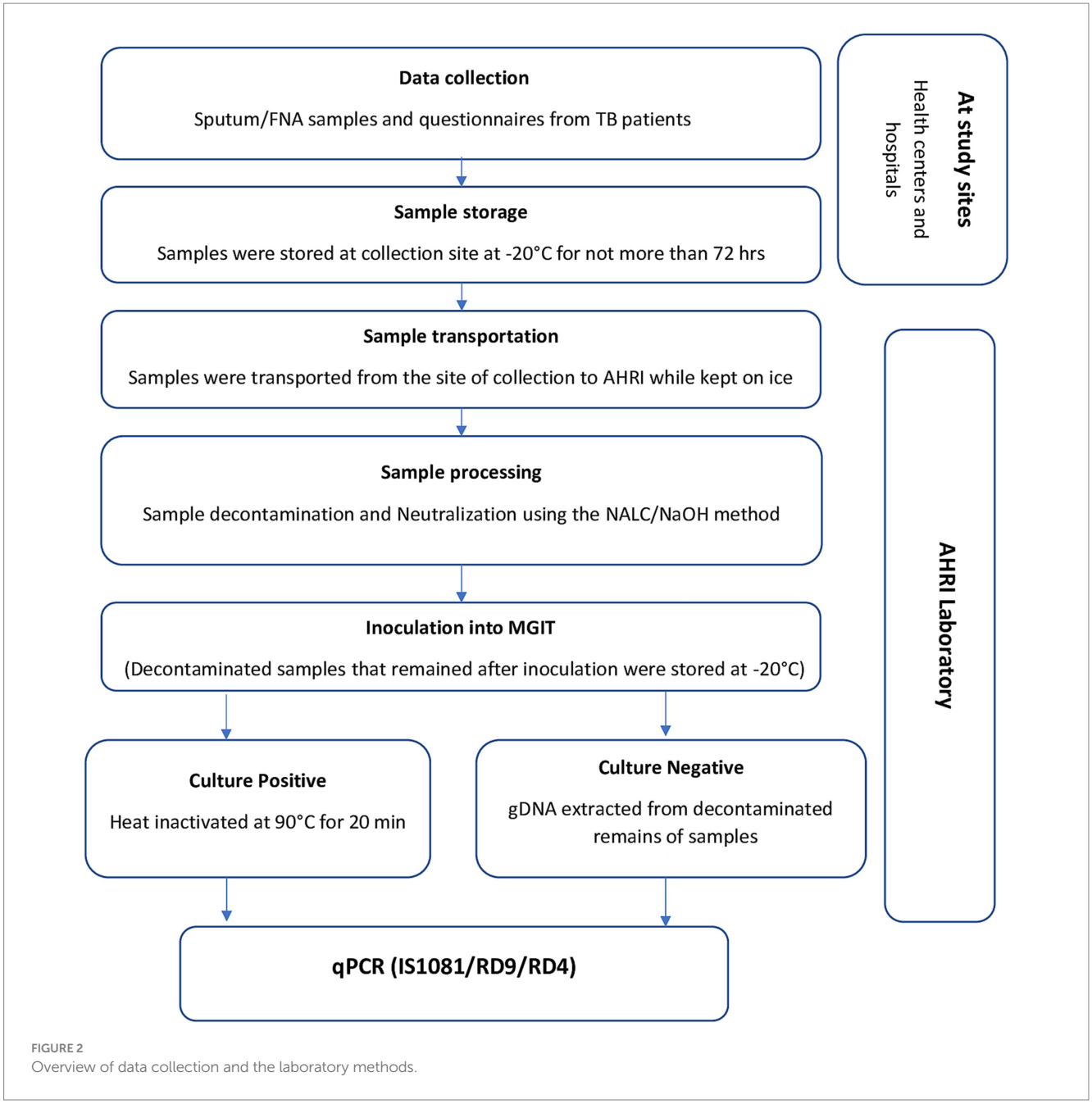

FIGURE 2
Overview of data collection and the laboratory methods.

specific primers for IS*1081* was used as a screening test to identify the presence of genomes from the MTBC in the samples. Samples that tested positive for IS*1081* were then tested by qPCR for the presence of RD9 and RD4, for further species identification. The assay for IS*1081* was based on a protocol published by Dykema et al. (23) and the assays for RD9 and RD4 were based on the protocols described by Halse et al. (24) and King et al. (25), respectively.

The reaction mixture for the IS*1081* assay was 10 µL PrimeTime Gene Expression Master Mix (2X) (Integrated DNA Technologies, Inc), 0.5 µL PrimeTime qPCR Assay (40X) consisting of premixed primers and probe of IS*1081*_F 5′-GGC TGC TCT CGA CGT TCA TC-3′; IS*1081*_*R* 5′-CGC TGA TTG GAC CGC TCA T-3′, IS*1081*_*P* [6FAM] CTG AAG CCG ACG CCC TGT GC [BHQ1], 4.5 µL nuclease free water, and 5 µL template DNA in a final volume of 20 µL. Reaction mixture for the RD4 assay was 2.5 µL of 10 µM RD4_FW 5′- TGT GAA TTC ATA CAA GCC GTA GTC G -3′, 2.5 µL of 10 µM RD4_Rev 5′- CCC GTA GCG TTA CTG AGA AAT TGC -3′, 0.5 µL 10 µM RD4_Probe [6FAM]-AGC GCA ACA CTC TTG GAG TGG CCT AC-[BHQ1], 12.5 µL of TaqMan® Environmental Master Mix 2.0 (Applied Biosystems, [Thermo fisher]), 2 µL nuclease free water and 5 µL template DNA in a final volume of 25 µL. A similar reaction mixture was used for the RD9 assay with primer/probe sequence as follows: RD9_FW 5′-TGC GGG CGG ACA ACT C-3′, RD9_Rev 5′-CAC TGC GGT CGG CAT TG-3′, RD9_Probe [Cy5]-AGG TTT CAC CTT CGA CCC-[BHQ2]. The PCR cycling conditions for the IS*1081* assay were 3 min at 95°C for enzyme activation, followed by 15 s at 95°C for denaturation and 1 min at 63°C for annealing and extension involving a total of 40 cycles.

RD4/RD9 assays were performed at 50°C for 2 min, followed by 95°C for 10 min and then 40 cycles of 95°C for 15 s and 58°C for 1 min at which the fluorescence acquisition was performed. All samples were tested in duplicate, and average IS1081 Ct values less than 37 were considered positive. The reactions were performed using a Rotor-Gene (RG-3000).

2.6. Quality control

Standard operational procedures for all laboratory tests were employed uniformly throughout the study. To prevent possible contamination of qPCR assays, sample preparation and DNA extraction, qPCR master mix preparation, and qPCR amplification were carried out in three separate rooms using dedicated laboratory coats, pipettes and sterile tips. Furthermore, purified gDNA of *M. tuberculosis* H_{37}Rv and *M. bovis* BCG and sterile molecular grade water were used as positive and negative controls in each qPCR round.

2.7. Data entry and analysis

All demographic and laboratory data collected were entered into a Microsoft Excel spreadsheet and verified. The questionnaire and laboratory data were linked by a unique identification code. SPSS statistical software version 27 (IBM Corp., Armonk, N.Y., United States) was used for analysis. Frequencies and cross-tabulation were used to summarize descriptive statistics. Bivariate and multivariable logistic regression analysis were applied to determine the significance among categorical variables. A *p*-value less than 0.05 was considered statistically significant.

2.8. Ethical considerations

The study was approved by AHRI/ALERT Ethics Review Committee (AAERC) (Ref. No: 301/001/2015). Study participants were provided with adequate information about the project and its commitments before signing informed consent.

3. Results

3.1. Characteristics of the study population

A total of 385 participants were enrolled in this cross-sectional study, including 287 PTB and 98 TBLN patients, stratified across six study sites as listed in Table 1 and mapped in Figure 1. The demographic analysis (Table 2) indicated that PTB was more frequent in males, while TBLN was more frequent in females ($p \leq 0.01$), with male-to-female ratios of 1.9:1 and 0.8:1, respectively. The mean age of all participants was 33 ± 14 years, while 270 (70%) of the study participants were in the age group between 20 and 45 years. With regards to consumption behavior of raw milk and raw meat among all interviewed patients, 64% of the respondents said that they consumed raw milk whereas 77% consumed raw meat; in total 52% of them consumed both raw milk and raw meat. There was no notable difference in milk consumption between patients of the two disease types, however, eating raw meat was significantly less common among the TBLN patients (OR = 0.4, CI 95% 0.2–0.7; $p < 0.01$). TBLN cases were more frequent among study participants who reported close contact with animals (OR = 2.3, CI 95% 1.3–3.9; $p < 0.001$) as compared to those who did not.

3.2. Culturing and typing of *Mycobacterium tuberculosis* complex

Culturing of mycobacteria from sputum and FNA samples using the MGIT system yielded 198 (68.9%) and 57 (58.2%) isolates, respectively. All culture positive samples were also positive for acid-fast bacilli as shown by ZN staining. Molecular typing by qPCR was performed on all 255 culture positive samples and all were first identified as MTBC by being positive for the IS*1081* assay. Among them, 249 (97.6%) samples had also a positive RD9 result (intact RD9 locus) and were subsequently classified as *M. tuberculosis*, while the remaining six culture positive MGIT samples (2.4%) were identified as both RD9 and RD4 deficient and thereby classified as *M. bovis*. Five out of these six *M. bovis* isolates originated from PTB patients whereas one was sampled from a TBLN patient (Table 3, Supplementary Table S1). All six patients identified with *M. bovis* infection in this study were males among whom four had occupations associated with animal handling (Table 4).

In attempts to identify the causative agents among TB patients with culture-negative results, gDNA was extracted from the remains of their sputum and FNA samples for 115 out of 130 patients and the extracted gDNA was used for molecular typing by qPCR. Thirty of these 115 samples (26.1%) were typed as *M. tuberculosis* as shown by IS*1081* being present and RD9 intact. Samples confirmed as positive for the IS*1081* assay, but with a negative result for the RD9 and RD4 assays, were classified as MTBC with no further characterization and accounted for 48/115 (41.7%) of the culture-negative cases. All of these samples had a Ct value between 31 and 37 for IS*1081* qPCR. The remaining 37 culture negative samples were negative also for IS*1081* by the qPCR assay (Supplementary Table S2). None of the patients with

TABLE 1 Number of enrolled pulmonary TB and TB lymphadenitis patients with clinical TB symptoms stratified by collection site and rates of culture-positivity.

Collection site	Pulmonary TB		TB Lymphadenitis	
	No of patients	Culture-positive	No of patients	Culture-positive
Adama	75	53 (70.7%)	32	14 (43.8%)
Bishoftu	166	116 (69.9%)	45	31 (68.9)
Sendafa	19	13 (68.4%)	0	0
Holeta	10	10 (100%)	0	0
Sebeta	17	6 (35.3%)	0	0
Addis Ababa	0	0	21	12 (57.1%)
Total	287	198 (69.0%)	98	57 (58.2%)

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TABLE 2 Demographic and clinical characteristics of the study participants from central Ethiopia ($n = 385$).

Patient characteristics		PTB N (%)	TBLN N (%)	Crude OR 95%CI	Adjusted OR 95%CI
Sex[a]	Male	188 (65.5%)	43 (43.9%)		
	Female	99 (34.5%)	55 (56.1%)	2.4 (1.5–3.9)***	2.0 (1.2–3.5)**
Age[b]	<20	27 (9.5%)	21 (21.4%)		
	20–45	207 (72.6%)	63 (64.3%)	0.4 (0.2–0.7)**	0.4 (0.2–0.9)*
	>45	51 (17.9%)	14 (14.3%)	0.4 (0.2–0.8)*	0.5 (0.2–1.1)
Previous TB history	No	237 (82.6%)	78 (79.6)		
	Yes	40 (13.9%)	14 (14.3%)	1.1 (0.6–2.1)	1.1 (0.6–2.4)
	Unknown	10 (3.5%)	6 (6.1%)	1.8 (0.6–5.2)	4.3 (1.1–16.2)*
History of BCG vaccination	No	177 (61.7%)	70 (71.4%)		
	Yes	54 (18.8%)	23 (23.5%)	1.1 (0.6–1.9)	1.3 (0.7–2.5)
	Unknown	56 (19.5%)	5 (5.1%)	0.2 (0.1–0.6)**	0.2 (0.1–0.5)***
Raw Milk consumption	No	100 (34.8%)	25 (25.5%)		
	Yes	175 (61.0%)	72 (73.5%)	1.6 (0.9–2.8)	1.6 (0.9–3.2)
	Unknown	12 (4.2%)	1 (1.0%)	0.3 (0.0–2.7)	0.5 (0.1–4.1)
Raw meat consumption	No	49 (17.1%)	34 (34.7%)		
	Yes	236 (82.2%)	62 (63.3%)	0.4 (0.2–0.6)***	0.4 (0.2–0.7)**
	Unknown	2 (0.7%)	2 (2.0%)	1.4 (0.2–10.7)	1.4 (0.2–14.1)
Level of contact with cattle	Not close	122 (42.5%)	24 (24.7%)		
	Moderate	33 (11.5%)	14 (14.4%)	2.2 (1.0–4.6)*	1.4 (0.6–3.4)
	Very close	132 (46.0%)	59 (60.8%)	2.3 (1.3–3.9)**	2.2 (1.2–4.1)*

[a]Adjusted for age and site alone.
[b]Adjusted for sex and site alone.
*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

TABLE 3 Molecular identification of disease agents by qPCR in culture-positive and culture-negative samples stratified by type of TB disease.

Samples processed for culture	Pulmonary TB (Sputum smear –) $N = 180$	Pulmonary TB (Sputum smear +) $N = 107$	TB lymphadenitis (FNA) $N = 98$	Total $N = 385$
Culture positive	93 (51.7%)	105 (98.1%)	57 (58.2%)	255 (66.2%)
Molecular typing	93	105	57	255
M. tuberculosis	90 (96.8%)	103 (98.1%)	56 (98.2%)	249 (97.6%)
M. bovis	3 (3.2%)	2 (1.9%)	1 (1.8%)	6 (2.4%)
Negative	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Culture negative	87 (48.3%)	2 (1.9%)	41 (41.8%)	130 (33.8%)
Molecular typing*	78	2	35	115
M. tuberculosis	24 (30.8%)	1 (50%)	5 (14.3%)	30 (26.1%)
M. bovis	0	0	0	0
MTBC	39 (50%)	1 (50%)	8 (22.9%)	48 (41.7%)
Negative	15 (19.2%)	0	22 (62.8%)	37 (32.2%)
Identification rate	86.7%	100%	71.4%	86.5%

MTBC, Mycobacterium tuberculosis complex species; FNA, fine-needle aspirate.
*Typing of a culture-negative sample was dependent on access to gDNA from the original processed sample.

culture-negative results but with IS*1081* positive results was identified as *M. bovis*, as determined by gDNA extraction and qPCR.

Considering all samples that were identified as MTBC by culture and subsequent qPCR typing, or by direct qPCR typing, the overall identification rate in this study was 86.5% with 100% identification for

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TABLE 4 Sociodemographic and clinical characteristics of the six study participants identified with *Mycobacterium bovis* infection.

Patient characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Gender	Male	Male	Male	Male	Male	Male
Age (yr)	13	38	23	38	46	34
Occupation	Student	Employee	Veterinarian	Animal attendant	Farmer	Meat seller
Location	Addis Ababa	Bishoftu	Sebeta	Sebeta	Bishoftu	Bishoftu
Previous TB history	No	No	No	No	No	No
Raw milk consumption	No	No	No	No	Yes	Yes
Raw meat consumption	No	No	No	Yes	Yes	Yes
Level of cattle contact	Not close	Not close	Very close	Very close	Very close	Very close
Type of TB disease*	TBLN	PTB+	PTB−	PTB−	PTB−	PTB+

*PTB+, Pulmonary TB smear+; PTB−, Pulmonary TB smear−; TBLN, TB Lymphadenitis.

smear-positive PTB, 86.7% for smear-negative PTB, and 71.4% for TBLN (Table 3).

4. Discussion

This study was designed to better understand the prevalence of zTB in humans living in central Ethiopia where bTB is highly prevalent in cattle. bTB has likely been endemic in Ethiopian cattle since records began nearly 50 years ago (26), and a high rate of bTB in the intensive dairy sector in central Ethiopia has been documented over at least the last 15 years (16–18), suggesting that the human population in this area has been highly exposed to bTB for a long time and that the risk of zTB is significant. In the present study, the overall *M. bovis* prevalence among 255 culture-positive cases was 2.4%, which is higher than previous reports from Ethiopia that have only reported a handful of *M. bovis* cases among much larger study populations, leading to estimated zTB rates far below 1% (19, 20). Interestingly though, these latter studies have, to a large extent, only explored human populations living in areas where extensive cattle husbandry is dominant, with mainly cattle of zebu breeds. As tuberculin testing and abattoir surveys of zebu cattle in these areas of Ethiopia have mostly reported relatively low bTB rates of 0–5% (14, 15) and rarely above 10% (15, 27), it is tempting to suggest that these lower rates in cattle lead to a lower risk of zTB transmission from the zebus which could explain the overall low zTB rates of <1% in Ethiopia (20). Local zebus appear to have higher resistance to bTB than exotic cattle breeds (28, 29). The introduction of exotic breeds and high-yield dairy systems into African nations was not without controversy because it may not have adequately taken ecological and cultural variations into account. In Africa, the dietary habit of the people, close physical contact between animals and humans, and inadequate bTB control in animals have facilitated transmission of the disease between animals and humans (30). In Ethiopia, the national herd of exotic Holstein-Friesian cattle has increased over many decades driven by the country's significant need for increased milk production in urban areas. However, higher risk of zTB has been advised, as a consequence thereof, due to intensive rearing of these exotic breeds that are likely more susceptible to bTB (29, 31). Approximately ten times higher bTB rates in cattle recorded in the dairy belt in central Ethiopia, which is dominated by exotic breeds, may correlate well with the significantly higher zTB prevalence in the human population (2.4%) living in that area, as we are reporting here. In fact, five of the six human cases in this study lived in Sebeta and Bishoftu, two sites that have recorded >70% bTB prevalence at herd level (17). Due to free cattle movement in Ethiopia and lack of a bTB control program, such as a test-and-movement regulation to avoid bTB infected cattle being dispersed, it is inevitable that bTB will spread from the central region to other regions with lower prevalence. This is in particular concerning the expansion of the intensive dairy sector to new urban centers across the country. A further shift to intensive dairy production with more exotic or cross-bred cattle, without any interventions, will likely increase the risk for zTB in the Ethiopian population.

Earlier publications on zTB in Ethiopia (19, 32) have tried to explain the extremely high rate of EPTB reported in Ethiopia (with regional variation between 20 and 45% and dominated by TBLN) by the national cattle herd being endemic for bTB and by subsequent transmission to humans through common raw milk and meat consumption. However, Berg et al. (32) concluded that bTB may contribute to the high EPTB rates, but that it is not the main factor. Their initial hypothesis that high endemic bTB rates in cattle would be reflected in the human population was largely based on historical figures on bTB. Epidemiological work during the first half of the 20th century showed that the agrarian societies in Europe suffered heavily from bTB in their national cattle herds, with average animal rates between 20 and 40% commonly reported (33), especially among the intensively reared dairy herds. In parallel, many European countries saw human TB incidence rates above 200/100,000 population (similar to those recorded in Ethiopia over the last few decades). Based on the methodologies available at that time for distinguishing between the two TB pathogens—*M. tuberculosis* and *M. bovis*—it was estimated that approximately 10–15% of TB in humans was caused by the bovine version of the TB bacilli (at that time not yet named *M. bovis*) (33, 34) through consumption of unpasteurised dairy products or interaction with infected cattle herds. This assessment was reinforced by the identification of the bTB version in humans to a higher degree among EPTB cases than among PTB cases (35, 36), suggesting transmission from cattle by ingestion of infected milk rather than aerosol transmission through inhalation of the bacilli. Despite similar circumstances that could lead to high zTB transmission, there might be several explanations why we cannot translate these epidemiological figures from Europe a century ago to the current situation in Ethiopia. One argument is that the ability to correctly identify the disease agent has improved. In Europe at that time, species identifications were mainly based on phenotypic characteristics of the disease agents, such as

differentiation by colony morphology and biochemical tests (37). These methods were less accurate and reproducible as compared to the current genotyping methods that have been developed after the genomic characterisations of all known species within the MTBC (2, 38). Therefore, the rates of zTB reported from Europe about a century ago may be less reliable, thereby making a comparison with current genotyping difficult. Since the present study used a combination of improved culturing yield through the MGIT system and a highly sensitive genotyping technique, the results that we report here are more likely to be closer to the true prevalence of *M. bovis* in humans in central Ethiopia.

Another question relates to the disease exposure of zTB among Ethiopians. A large proportion of our study population had a habit of consuming untreated milk (64%) and raw meat (77%). In many Ethiopian communities, untreated (raw) milk is consumed by many, mainly because of its accessibility and lower price but some also find untreated milk having a better taste. Earlier studies have documented that 35–50% of the society in Ethiopia frequently drinks raw milk in its fresh form (39, 40), while a more recent study in central Ethiopia reported 90% of the participants boiling their milk before consumption (41), a difference that could indicate a considerable change in milk consumption habits during the last few years. In addition to raw milk, research has shown that Ethiopians consume a form of fermented milk (locally named *ergo*) at significant rates (41, 42). Under specific conditions, fermented and soured milk has been shown to suppress the growth of *M. bovis* in milk (43, 44), albeit not as successfully as the process of milk pasteurization, which should abolish the risk of zTB in milk. Additionally, a significant proportion of the population in Ethiopia still consumes raw meat (41, 45). Although there were too few zTB cases to draw any strong conclusions on, three out of six participants infected with *M. bovis* in the current study consumed either raw meat or raw milk. Ethiopians' practice of consuming raw meat and milk can foster favorable conditions for zTB, especially in places where bTB is highly prevalent. However, as discussed above, the high rate of TBLN recorded in Ethiopia cannot be explained by high zTB transmission alone (32).

A significant part of the population in Ethiopia works in the dairy sector. All *M. bovis* cases in the current study were isolated from male study participants. Four out of six *M. bovis* cases had close contact with cattle, likely linked with their occupational status as a veterinarian, an animal attendant, a farmer, or a meat seller. As these occupations are male dominated, this might also explain the absence of female *M. bovis* cases. It has been documented that the risk of zTB increases in areas where bTB is endemic in cattle and where people live under conditions that favor direct contact with infected animals and/or with untreated animal products (7, 46). Therefore, although the risk of contracting zTB is not limited to people working with animals, promoting the use of personal protective equipment by farmers, veterinarians, and abattoir workers may help to reduce the risk of exposure while having contact with *M. bovis*-infected animals. On the other hand, two *M. bovis*-infected cases in this study reported neither an occupational risk nor a consumption habit of untreated milk or raw meat. Although such exposure could still have been possible, these two cases could also have been the result of person-to-person transmission cycles of *M bovis* as previously documented by others (47) or cases of latent infection of this zoonotic pathogen. It is interesting to note that five out of six *M. bovis* infected cases in the current study had PTB and two of them had positive smear results (1+ and 3+), suggesting that *M. bovis* can generate high bacterial loads in sputum, which could have an impact on onwards disease transmission.

A more recent development concerning zTB risks refers not only to *M. bovis* being associated with zTB but that other sub-species of the MTBC may also infect cattle (10) and subsequently transmit to humans. In India, *M. bovis* is rarely found in cattle while *M. orygis* and *M. tuberculosis* are frequently isolated (48, 49). We and others have also isolated *M. tuberculosis* from cattle in Ethiopia (50–52), which is likely to be a spillover from TB in humans (reverse zoonosis). It has been shown experimentally that *M. tuberculosis* is less prone to cause pathology in cattle than *M. bovis* (53, 54), but whether *M. tuberculosis* infected cattle play a significant role in zTB transmission to humans still remains to be answered.

A limitation of this study is the qPCR assays, which are limited in their ability to differentiate between MTBC members other than *M. tuberculosis* and *M. bovis*. Also, the IS*1081* assay is expected to be more sensitive than the RD9 and RD4 assays since it is a multiple copy gene (22). Therefore, samples positive for IS*1081* but negative for RD9 and RD4 by qPCR were likely to have a low bacterial load and consequently a gDNA concentration of MTBC below the limit of detection for the RD9 and RD4 assays. Samples identified only by IS*1081* can then still be either *M. tuberculosis* or *M. bovis*, or even another type of MTBC, affecting the overall prevalence figures presented in this study.

5. Conclusion

This Ethiopian study documented a 2.4% prevalence of *M. bovis* in humans, which is higher than those reported in previous large zTB studies in Ethiopia; and we suggest that the higher bTB rates recorded in the dairy sector in central Ethiopia have likely had an impact on the zTB rate in the same area. However, despite sampling in an area with very high bTB prevalence in cattle, the rate of *M. bovis* in this study is still far from the zTB rates reported from Europe at its bTB endemic peak about a century ago. MTBC speciation tools that we used may largely explain this difference. Occupational risk and the widespread habit of raw animal product consumption were noted as possible sources of *M. bovis* infection in humans, while isolation of *M. bovis* from PTB patients also suggests the potential for human-to-human transmission, especially in patients with no known contact history with animals. There is a high chance that patients with *M. bovis* infection get ineffective TB treatment, as *M. bovis* is naturally resistant to pyrazinamide, a first-line drug used for treatment of TB. MTBC species identification should be encouraged, particularly for patients with relapse and treatment failure. New molecular TB diagnostic approaches in the pipeline should be able to differentiate *M. tuberculosis* from *M. bovis* to warrant improved patient management concerning treatment regimens. However, TB control programs need to take into account the additional cost of differential diagnostics in light of the relatively low global burden of zTB. In parallel, stricter adherence to heat-treatment of milk, proper meat inspection, and increased public awareness on the dangers of consuming raw animal products when it comes to zoonotic diseases in general, and zTB in particular, is crucial. Special attention should be given to the occupational risks within the livestock sector, especially in areas where high prevalence of bTB in cattle is well documented.

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 studies involving humans were approved by AHRI/ALERT Ethics Review Committee (AAERC). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

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Author contributions

AM, JW, and SB conceived and designed the study. BT, GH, SC, FM, RA, and SA sample collection and laboratory analysis. AM, SA, and SB performed data curation and data interpretation. SA wrote the first draft of the manuscript. AA, AM, JW, RA, SA, and SB reviewed the manuscript. 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/fpubh.2023.1204525/full#supplementary-material

SUPPLEMENTARY TABLE S1
qPCR result for culture positive samples.

SUPPLEMENTARY TABLE S2
qPCR results for extracted sputum and FNA (Culture negatives).

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Data analysis of zoonoses notifications in Aboriginal and Torres Strait Islander populations in Australia 1996–2021: implications for One Health

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Introduction: Zoonoses are a health concern for Aboriginal and Torres Strait Islander peoples in Australia that face elevated risk of disease related to the environment and animals. Internationally, One Health is encouraged to effectively manage zoonoses by taking integrated approaches involving animal, human, and environmental health sectors to improve health outcomes. However, Australia's health systems manage zoonotic diseases in animals and people separately which does not support a One Health approach. For the effective management of zoonoses, a strong evidence base and database regarding the epidemiology of zoonotic pathogens is needed. However, we currently lack this evidence limiting our understanding of the impact of zoonoses on Aboriginal and Torres Strait Islander populations.

Methods: As a first step towards building the evidence base, we undertook a descriptive analysis of Aboriginal and Torres Strait Islander zoonotic notifications in Australia from 1996 to 2021. We presented notifications as annual notification rates per 100,000 population, and percentages of notifications by state, remoteness, sex, and age group.

Results: Salmonellosis and campylobacteriosis were the most notified zoonoses with the highest annual notification rates of 99.75 and 87.46 per 100,000 population, respectively. The north of Australia (Queensland, Northern Territory and Western Australia), remote and outer regional areas, and young children (0–4 years of age) had the highest percentages of notifications.

Discussion: To our knowledge, these findings are the first national presentation of the epidemiology of zoonoses within Aboriginal and Torres Strait Islander populations. A greater understanding of transmission, prevalence and impact of zoonoses on Aboriginal and Torres Strait Islander peoples (including animal and environmental health factors) is required to inform their effective management through a One Health approach.

KEYWORDS

Aboriginal and Torres Strait Islander, animal health, environment, One Health, public health, zoonoses

1. Introduction

Zoonotic pathogens are a global health concern and can cause a threat to human health particularly where animals and humans live closely together. Zoonoses are diseases caused by pathogens that can be transmitted between animals and people through various avenues including airborne, vectors, direct or indirect contact, food borne, water borne, and soil borne transmission (1). Both domestic and wild animals can be involved in the transmission of zoonotic pathogens, with environmental exposure, such as via vectors, also a key component in many situations. Therefore, understanding relationships and interactions between animal, human, and environmental health is integral to understanding transmission pathways and addressing the risk of zoonoses.

Globally, there has been an increase in emerging zoonotic diseases with 60% of all infectious diseases in humans and 75% of emerging infectious diseases in humans of zoonotic origin and commonly originating from wildlife (2, 3). An example of an emerging infectious disease is SARS-CoV-2 which is responsible for the recent COVID-19 pandemic and is hypothesised to have originated from wild animals with the impacts on health felt globally (4, 5). Following an increase in zoonotic outbreaks, strong support for countries to adopt integrated health approaches has been recommended including focusing on environmental and animal management as part of public health responses to control disease (6, 7). Endemic diseases (including neglected zoonotic diseases) are also a great concern for low resourced communities, many of which have high Indigenous populations and can be at higher risk of zoonoses (8). Whilst zoonotic diseases are a risk for Indigenous communities, they are also among the most under-diagnosed diseases in humans with the full burden of disease not well understood (8).

Aboriginal and Torres Strait Islander peoples consist of an estimated 984,000 people across some 300 different language groups, making up approximately 3.8% of the Australian population (9). Whilst improvements in Aboriginal and Torres Strait Islander health are a focus of national policy within Australia (10), there continue to be large inequities in health outcomes and access to health care (11). Aboriginal and Torres Strait Islander peoples are disproportionately affected by diseases related to environmental health, including communicable diseases, with further disparities experienced in remote areas (12, 13). Environmental health factors contributing to this include quality of housing, water, air, sanitation, disease control, and food and water safety (12). The impact of these factors is hypothesised to increase due to the changing climate, which in tropical regions of Australia may lead to a range of negative sequalae including increased risk of vector borne zoonotic pathogens (14). Animal and human health factors, along with social determinants of health, also contribute to these disparities, for example remote areas have higher domestic animal populations with people and animals living closely together, many without access to animal health care or associated services (15–18). Communities in the north of Australia are at increased risk of exotic zoonotic pathogens that are present in neighbouring countries with surveillance vital to detection and disease control (19). Interactions with wild and feral animals can also increase risk of disease, for example hunting can be common practise in some communities increasing interactions between people, domestic, and wild animals (20). Due to the elevated risk of zoonoses in communities, animal health programmes can be beneficial in improving health outcomes and increasing awareness (21, 22), however, how to operationalise a One Health approach needs further consideration.

Within Australia, nationally notifiable diseases in people are those that have been assessed as a public health priority and meet multiple assessment criteria, including importance for Indigenous health (23). Nationally notifiable diseases are reportable to state and territory health authorities, with data supplied to the National Notifiable Diseases Surveillance System (NNDSS). The NNDSS includes the surveillance of more than 50 communicable diseases of national public health importance and is managed by the Australian Government Department of Health and Aged Care, with oversight provided by the Communicable Diseases Network Australia (24). This surveillance system helps with the monitoring, detection, and control of communicable diseases and informs the coordination of outbreak responses (25). Notifiable diseases disproportionately affect Aboriginal and Torres Strait Islander people accounting for 8.4% of all notifications from 1991 to 2011 (26). Notifications among Aboriginal and Torres Strait Islander populations are, however, thought to be an underestimation with Indigenous status commonly under reported (26). Whilst many diseases included in the NNDSS are of animal origin, the database only includes disease that is diagnosed in people.

Internationally, the effective management of zoonoses is seen as a priority by leading health organisations and a One Health approach is strongly encouraged at a global, national, and local level (27). One Health is an interdisciplinary approach to health recognising that the "health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and inter-dependent" (pg 2, 28). Zoonotic programmes that take a One Health approach and involve multiple sectors have been found to be more effective in reducing disease than those within a single sector (28). Zoonotic programmes targeting specific diseases may also require additional management approaches due to differing transmission pathways. The One Health approach can assist in understanding and addressing the factors that lead to the increased risk of communicable disease within Aboriginal and Torres Strait Islander populations. One Health aligns with Aboriginal and Torres Strait Islander cultures and knowledge's that recognise these integral health relationships and support holistic approaches to health care (29). The underlying principles of One Health highlight the need for equity between sectors, inclusion and engagement of communities, transdisciplinary approaches, and acknowledging the role of the environment in health (30). It can also assist with timely and effective public health responses, accurate decision making, accountability, and shared responsibilities and resources (31, 32).

The development of joint human and animal health systems has been recommended for effective management of zoonoses, including data sharing and integrated surveillance systems (8). However, many countries do not have adequate mechanisms in place for managing zoonoses across human and animal health sectors limiting the ability to prevent and control disease (8, 31). Australia's human and animal health systems are managed separately with limited communication between sectors (33). Therefore, the management of zoonoses is managed separately with notifiable pathogens and subsequent disease not consistent between databases or between states and territories with some zoonoses nationally notifiable in people but not animals [such as Q fever (also known as coxiellosis)]. National notifiable disease lists commonly focus on zoonoses related to livestock and

wildlife due to economic and trade implications however, there is limited surveillance of zoonotic pathogens related to companion animals with dogs and cats common in communities. Some zoonoses of public health importance are also not nationally notifiable in people and animals, such as *Strongyloides* however, Strongyloides Australia has recommended its addition to the national notifiable list due to high rates in Aboriginal communities (34).

Therefore, the current approach does not adequately account for the impact of animal and environmental factors that contribute to human health outcomes, limiting our ability to improve the management of zoonoses (35, 36). It also leads to challenges in the identification, prevention, and control of zoonotic pathogens. Australia's health system could benefit from an integrated national system, assisting in timely detection and effective management of zoonoses (33). Examples of this can be seen internationally in Denmark and Canada where they have taken an integrated and continuous approach to monitoring antimicrobial resistance in animals and people, however it can be argued that these systems fall short of enacting a true One Health approach (37, 38). Indigenous governance and leadership in health systems is also commonly limited and needs further consideration to strengthen and inform disease management (39).

Zoonotic pathogens are of increasing concern globally and they are commonly under-reported and neglected, with many gaps in our understanding of them within Aboriginal and Torres Strait Islander populations. To address this and inform a One Health approach to the management of zoonoses, we initially undertook a scoping review regarding zoonoses in Aboriginal and Torres Strait Islander populations and found many gaps within the evidence base (40). This study builds on these findings by investigating the epidemiology of notifiable zoonoses within Aboriginal and Torres Strait Islander populations in Australia.

2. Materials and methods

This study was conducted with approval from the Australian Institute of Aboriginal and Torres Strait Islander Studies (AIATSIS) Research Ethics Committee (EO243-20210406) and was undertaken by an Aboriginal-led and multidisciplinary research team.

2.1. Study design

Due to the current systems limitations, it was not possible to perform an integrated data analysis using animal and human health datasets due to differences in data access processes, data fields, and Indigenous identifiers. Therefore we limited our focus to the NNDSS and reported zoonoses in people.

We utilised the NNDSS database to undertake a descriptive analysis of zoonotic notifications in Aboriginal and Torres Strait Islander populations from 1996 to 2021. We aimed to understand the characteristics of notifications in the population by time, location, and demographic factors. First, the NNDSS disease list was assessed for zoonoses by two authors independently (TR, BC) with zoonoses of interest discussed and agreed. The zoonoses of interest included those that actively transmit between animals and people and did not include those that have a zoonotic origin but are now maintained through human-to-human transmission. A data request was then made to the Australian Government Department of Health and Aged Care for data access of the NNDSS database from 1996 to 2021 (24). The data analysis plan was developed with input from three authors (TR, RL, and JT) and analysis undertaken by two authors (TR as main analyst, JT as secondary analyst).

2.2. Statistical analysis

We performed a descriptive analysis of the NNDSS data from 1996 to 2021 to understand characteristics of zoonotic notifications within Aboriginal and Torres Strait Islander populations. The zoonoses included in the analysis were Ross River virus, brucellosis, campylobacteriosis, leptospirosis, listeriosis, ornithosis, Q fever, rabies, salmonellosis, viral haemorrhagic fever, Barmah Forest virus, Murray Valley encephalitis virus, Shiga toxin-producing *Escherichia coli* (STEC), anthrax, Japanese encephalitis virus, Kunjin virus, cryptosporidiosis, Australian bat lyssavirus, tularaemia, avian influenza, and Middle East respiratory syndrome. The variables used in the analysis included notification date received, state, statistical area level 3 (SA3s), disease code, age group, and sex for those notifications identified as Indigenous. Data were analysed in Stata 17 and Excel.

Aggregated data were used to calculate percentages of each zoonoses notified by Indigenous status nationally. We calculated notification rates per 100,000 Aboriginal and Torres Strait Islander population using aggregated annual notification data and estimated resident population data sourced from the Australian Bureau of Statistics (41). Notification rates were displayed graphically and zoonoses with rates less than 1 per 100,000 population removed. The notification rates were calculated as follows:

$$\text{Annual notification rate} = \left(\left[\text{number of cases notified} \,/\, \text{population size}\right]^{*} 100,000\right)$$

Aboriginal and Torres Strait Islander notifications are presented by state [Australian Capital Territory (ACT), New South Wales (NSW), Queensland (QLD), Western Australia (WA), Northern Territory (NT), Tasmania (TAS), Victoria (VIC), and South Australia (SA)], by sex (female, male) and by age group (0–4, 5–14, 15–29, 30–49, and 50+ years). Remoteness was derived using SA3s to group notification locations into the standard Australian Bureau of Statistics remoteness categories including major cities, inner regional, outer regional, remote and very remote (42). For state, remoteness, sex, and age group, percentages were calculated and displayed in bar charts. Missing data were included in results tables but were excluded from graphs.

3. Results

From 1996 to 2021, there were 29,786 Aboriginal and Torres Strait Islander zoonotic notifications, accounting for 3.1% of all zoonotic notifications in Australia. There were no notifications for rabies, viral haemorrhagic fever, anthrax, Australian bat lyssavirus, tularaemia, avian influenza, and Middle East respiratory syndrome.

Notifications were more common in Queensland (31.1%) and the Northern Territory (30.3%), and over half of all notifications were in remote (33.4%) and outer regional (29.9%) areas of Australia. The distribution of notifications in males and females was similar and those aged 0–4 years made up half of all notifications (50.8%; Table 1).

The zoonoses with the highest percentage of Aboriginal and Torres Strait Islander notifications were Murray Valley encephalitis (36.8%), followed by Kunjin virus (10.9%), Japanese encephalitis virus (8.3%), and cryptosporidiosis (7.5%; Figure 1; Supplementary Table 1).

The zoonoses with the highest annual notification rates (per 100,000 population) were salmonellosis (99.75), campylobacteriosis (87.46), cryptosporidiosis (61.14), Ross River virus (29.85), Barmah Forest virus (15.45), Q fever (6.39), STEC (4.25), and leptospirosis (2.88). All other diseases had annual rates of 1 or less per 100,000 population (Figure 2; Supplementary Table 2).

Leptospirosis (84.0%), brucellosis (75.0%), and Barmah Forest virus (57.4%) had high percentages of notifications in Queensland. Similarly, 68.0% of ornithosis cases were in New South Wales, 57.1% of Murray Valley encephalitis cases were in the Northern Territory, and 53.3% of STEC cases were in South Australia. Half of all Kunjin virus cases were in Western Australia, and half the Japanese encephalitis cases were in the Northern Territory and Queensland (Figure 3; Supplementary Table 3A).

Kunjin virus (83.3%), Murray Valley encephalitis (66.7%), and STEC (56.2%) had high percentages of notifications in remote areas. Similarly, majority of leptospirosis (84.0%) and Barmah Forest virus (54.2%) were in outer regional areas, with half the Japanese encephalitis notifications in remote areas and half in outer regional areas (Figure 4; Supplementary Table 3B).

Leptospirosis (88.8%), brucellosis (87.5%), Q fever (76.1%), and ornithosis (60.0%) had majority of notifications in males. On the other hand, Kunjin virus (66.7%), STEC (62.7%), Ross River virus (62.4%), and listeriosis (61.7%) had majority of notifications in females. The other zoonoses presented in similar percentages in both males and females (Figure 5; Supplementary Table 3C).

Cryptosporidiosis (84.7%), salmonellosis (56.4%), Murray Valley encephalitis (52.4%), and campylobacteriosis (48.9%) had high percentages in 0–4 years. Similarly, majority of Kunjin virus (83.3%) were in 15–29 years, half of Japanese encephalitis were in 5–14 and 30–49 years, and 51.1% of listeriosis notifications were in 50+ years of age (Figure 6; Supplementary Table 3D).

TABLE 1 Summary table of Aboriginal and Torres Strait Islander zoonoses notifications in Australia from 1996 to 2021.

Characteristics	Female *n* (%)	Male *n* (%)	Missing *n* (%)	Total *n* (%)
State				
ACT	48 (0.3)	48 (0.3)	0 (0.0)	96 (0.3)
NSW	1,739 (11.7)	1,956 (13.1)	4 (40.0)	3,699 (12.4)
NT	4,547 (30.7)	4,480 (30.0)	2 (20.0)	9,029 (30.3)
QLD	4,563 (30.8)	4,708 (31.5)	0 (0.0)	9,271 (31.1)
SA	717 (4.8)	705 (4.7)	0 (0.0)	1,422 (4.8)
TAS	68 (0.5)	58 (0.4)	0 (0.0)	126 (0.4)
VIC	267 (1.8)	269 (1.8)	2 (20.0)	538 (1.8)
WA	2,869 (19.4)	2,734 (18.3)	2 (20.0)	5,605 (18.8)
Total	14,818 (100.0)	14,958 (100.0)	10 (100.0)	29,786 (100.0)
Remoteness				
Major cities	2,170 (14.6)	2,246 (15.0)	4 (40.0)	4,420 (14.8)
Inner regional	1,038 (7.0)	1,046 (7.0)	2 (20.0)	2,086 (7.0)
Outer regional	4,382 (29.6)	4,521 (30.2)	3 (30.0)	8,906 (29.9)
Remote	4,974 (33.6)	4,971 (33.2)	1 (10.0)	9,946 (33.4)
Very remote	1,376 (9.3)	1,417 (9.5)	0 (0.0)	2,793 (9.4)
Missing	878 (5.9)	757 (5.1)	0 (0.0)	1,635 (5.5)
Total	14,818 (100.0)	14,958 (100.0)	10 (100.0)	29,786 (100.0)
Age group				
0–4 years	6,980 (47.1)	8,147 (54.5)	2 (20.0)	15,129 (50.8)
5–14 years	1,044 (7.1)	1,216 (8.1)	0 (0.0)	2,260 (7.6)
15–29 years	2,091 (14.1)	1,872 (12.5)	4 (40.0)	3,967 (13.3)
30–49 years	2,647 (17.9)	2,100 (14.0)	2 (20.0)	4,749 (15.9)
50+ years	2,053 (13.9)	1,618 (10.8)	2 (20.0)	3,673 (12.3)
Missing	3 (0.02)	5 (0.03)	0 (0.0)	8 (0.03)
Total	14,818 (100.0)	14,958 (100.0)	10 (100.0)	29,786 (100.0)

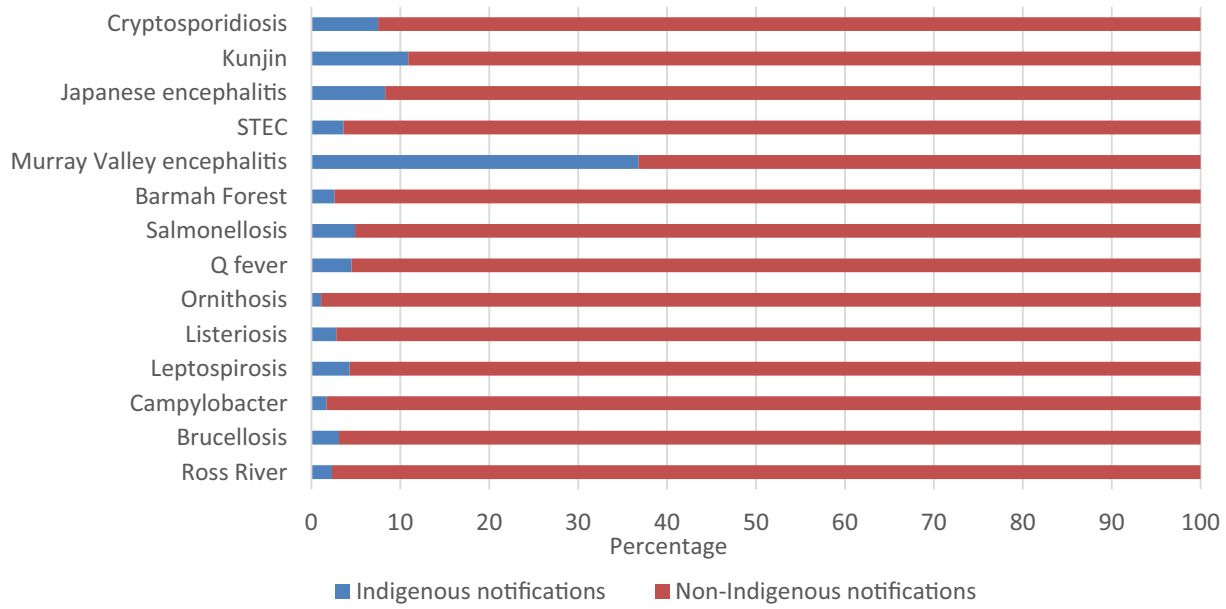

FIGURE 1
Percentage of zoonoses notifications by Indigenous status 1996–2021.

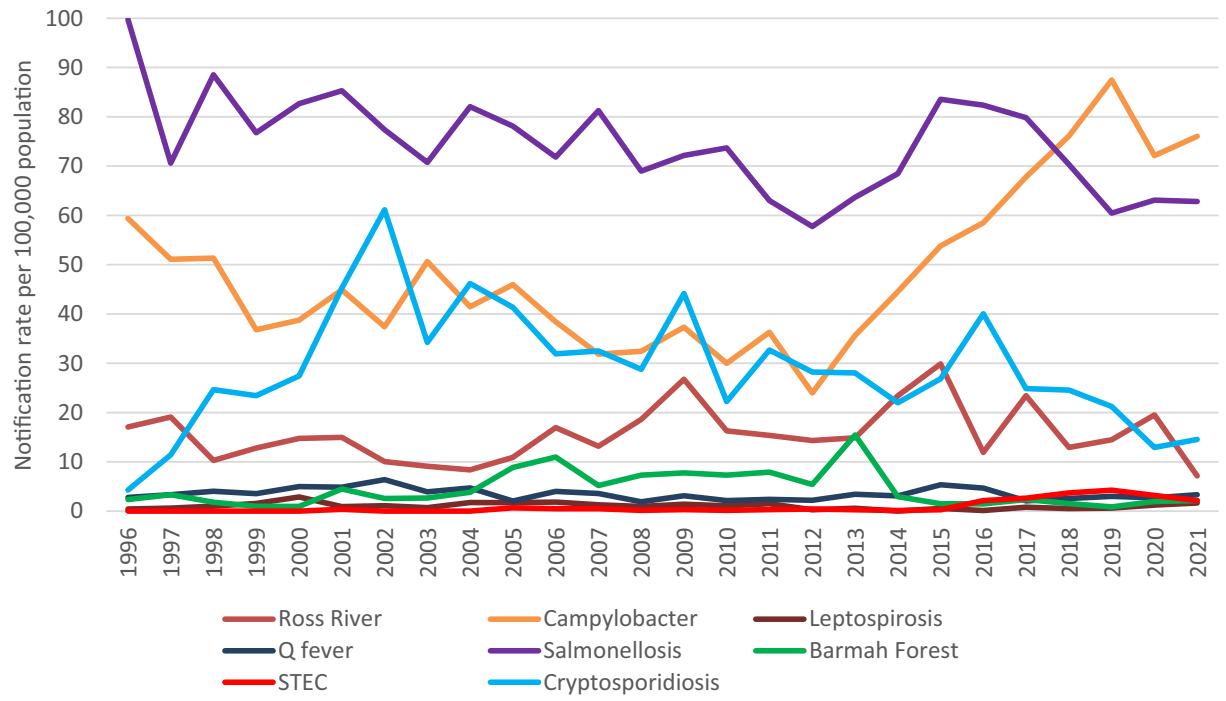

FIGURE 2
Aboriginal and Torres Strait Islander zoonoses notification rates per 100,000 population 1996–2021.

4. Discussion

4.1. Summary results

Just over 3% (3.1%) of all zoonotic notifications reported to the NNDSS from 1996 to 2021 were reported to be Aboriginal and Torres Strait Islander people. The percentage of Aboriginal and Torres Strait Islander zoonotic notifications was broadly similar to the overall population of 3.8% (9). However, Indigenous status is commonly underreported with a historical analysis identifying over half of all notifications to the NNDSS do not report Indigenous status (43). As these notifications are included as non-Indigenous, Aboriginal and Torres Strait Islander zoonotic notifications are likely to

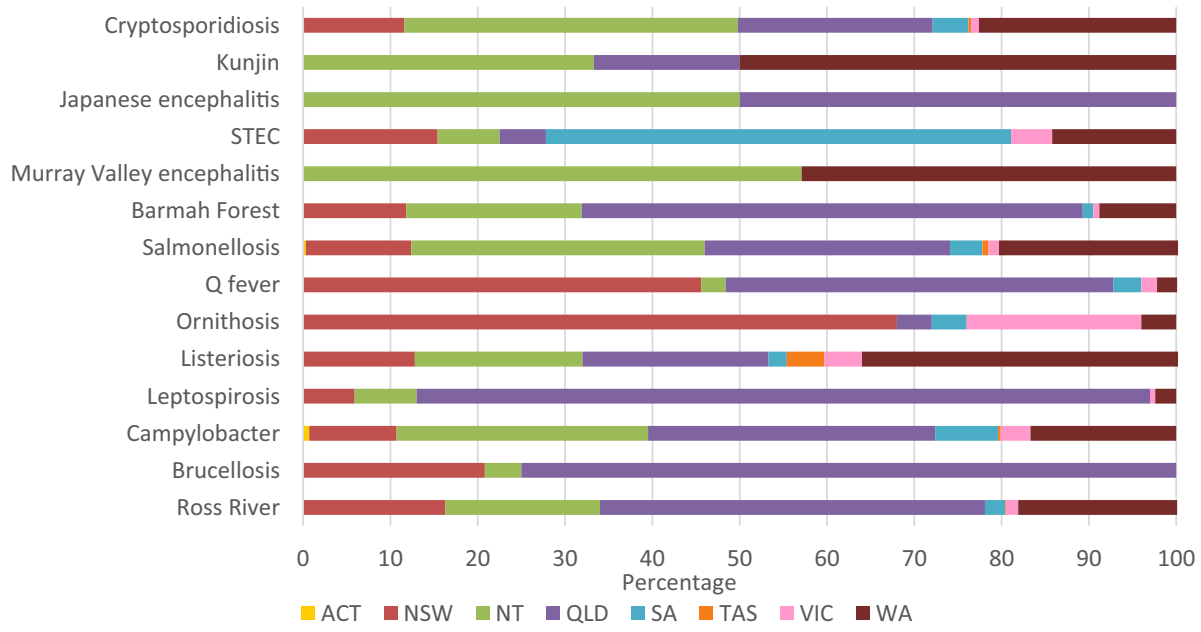

FIGURE 3
Aboriginal and Torres Strait Islander zoonoses notifications (%) by state 1996–2021.

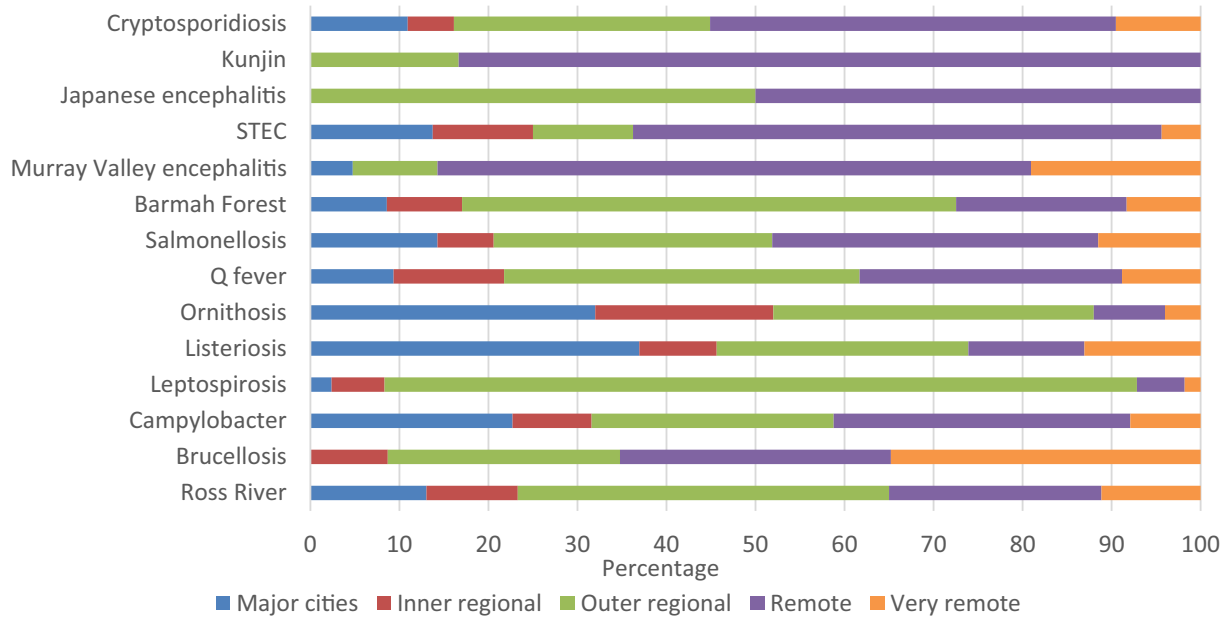

FIGURE 4
Aboriginal and Torres Strait Islander zoonoses notifications (%) by remoteness 1996–2021.

be underrepresented. Also, the barriers in accessing health care in many communities limit the ability to understand the true impact of zoonoses.

The highest percentages of zoonotic notifications were Murray Valley encephalitis, followed by Kunjin virus, and Japanese encephalitis however, when looking at annual notification rates all these diseases had rates less than 1 notification per 100,000 population per year. Alternatively, the zoonoses with the highest annual notification rates were salmonellosis, campylobacteriosis, and cryptosporidiosis. The zoonoses that did not have any notifications were rabies, viral haemorrhagic fever, anthrax, Australia bat lyssavirus, tularaemia, avian influenza, and Middle East respiratory syndrome.

Notifications were broadly in line with the percentage of the population in each state and territory with Queensland, Northern Territory, Western Australia, and New South Wales having the highest

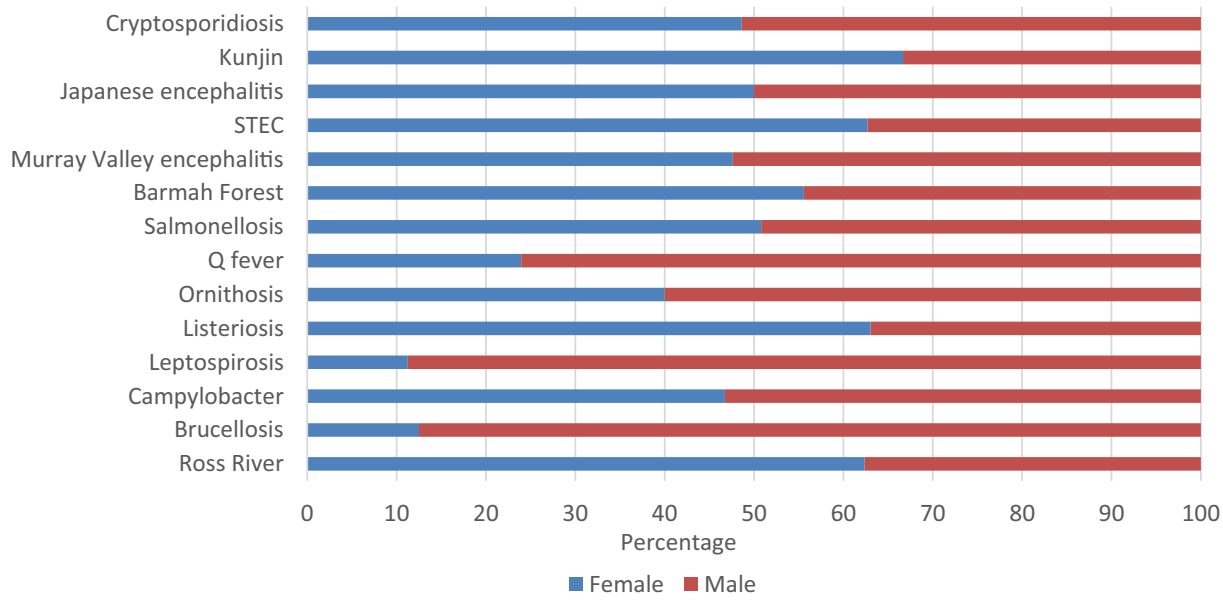

FIGURE 5
Aboriginal and Torres Strait Islander zoonoses notifications (%) by sex 1996–2021.

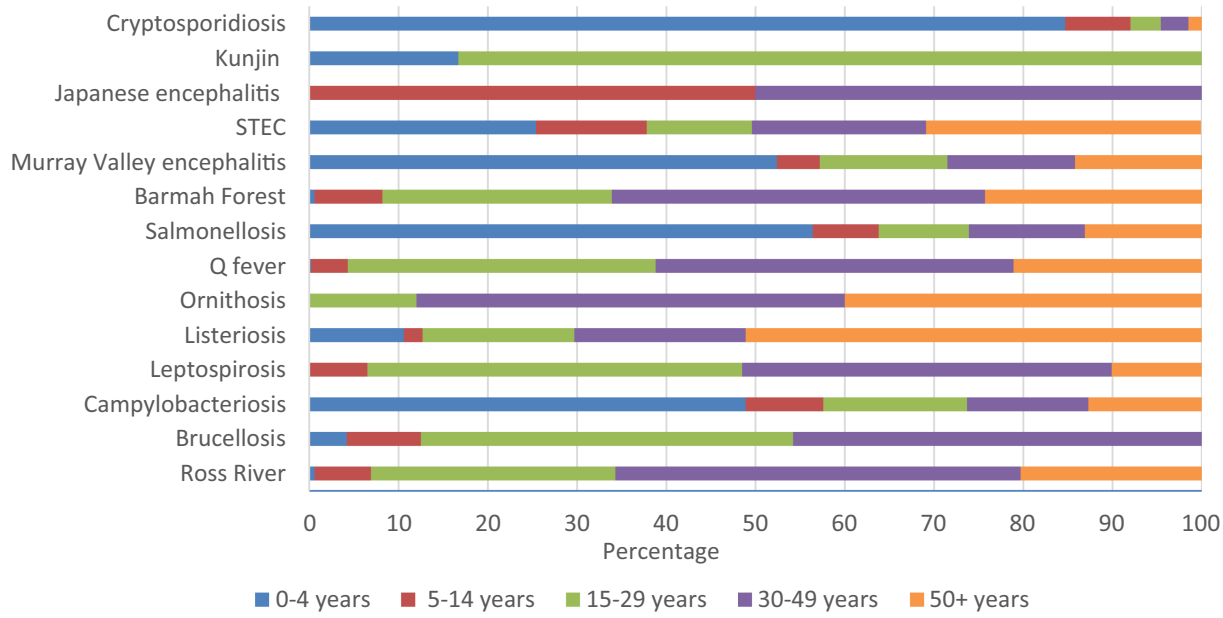

FIGURE 6
Aboriginal and Torres Strait Islander zoonoses notifications (%) by age group 1996–2021.

percentage of notifications and the Australian Capital Territory having the lowest (9). The most common remoteness category reported was remote areas of Australia which is a concern, as 18% of Aboriginal and Torres Strait Islander peoples live in remote and very remote areas (44) and these areas face higher risk of communicable diseases and limited access to health care (12, 13).

The percentage of total notifications by sex was similar. There are occupational exposures related to some zoonoses such as leptospirosis, brucellosis, and Q fever, all of which were more common for males and common exposures can include working with animals and animal products (45). Over half of all notifications were for children aged 0–4 years, with cryptosporidiosis and salmonellosis common in this age group. Whilst these zoonoses were the most common, they can also present with vague clinical signs and may not be identified therefore, results may be underrepresented. The Aboriginal and Torres Strait Islander population has a young age structure with one-third of the population under 15 years of age therefore, a higher level of notifications in young people would be expected (9).

The commonality of zoonoses notified for the north of Australia, for remote areas, and for young children may be due to an increased exposure to vectors and animals that carry and transmit disease. The north of Australia faces a tropical climate with vectors, such as mosquitos, common and the risk of vector borne zoonoses likely to increase due to a changing climate (14). Remote areas also have higher domestic animal populations and increased exposure where people and animals live closely together, many without access to animal health care or associated services (18). Therefore, the risk of zoonoses to people living in these areas may be higher. Similarly, communities can face increased environmental exposures due to a lack of appropriate housing and infrastructure, sanitation facilities, air quality, and food and water sources, increasing the risk of transmission and disease (12, 46). The most common zoonoses notified are usually food borne, highlighting the importance of appropriate food handling, storage, and food security, with food insecurity disproportionately experienced in Aboriginal and Torres Strait Islander communities, particularly in remote areas (47). Another factor that may explain higher notifications in remote areas is hunting activities which exposes people and domestic animals to wild animals that can carry disease (20).

These findings highlight that improving zoonoses prevention strategies within Aboriginal and Torres Strait Islander populations using One Health approaches, particularly for the north of Australia, for remote areas, and for young children, should be prioritised to reduce zoonotic notifications in the population. It is also important to consider zoonoses in the overall context of burden of disease within Aboriginal and Torres Strait Islander populations. To enact a One Health approach, strategies should acknowledge and address all One Health sectors and increase awareness about the transmission and risk of zoonoses including animal and environmental health exposures.

4.2. Strengths and limitations

To our knowledge, this is the first comprehensive national study of Aboriginal and Torres Strait Islander zoonotic notifications. This project was undertaken by an Aboriginal-led multidisciplinary research team with findings contributing to our understanding of zoonoses in the population whilst also highlighting gaps in the current system. The findings have highlighted areas of high notifications including specific diseases (salmonellosis and campylobacteriosis), young children (0–4 years of age), remote and outer regional areas, and the north of Australia (Queensland, Northern Territory, Western Australia). Additionally, we have identified gaps in our understanding of the true impact of zoonoses on the population and subsequent management.

Due to the current systems limitations, we reduced our focus to the NNDSS and reported zoonoses in people. This limited our ability to analyse the animal and environmental health factors that contribute to zoonotic notifications in people. Also, as we focused on notifiable zoonoses, we were not able to analyse zoonoses that may be prevalent in the population but are not considered notifiable. In interpreting the data, it is critical to recognise that notification data are based on diagnostic testing therefore, areas facing limited health care and laboratory diagnostic capacity may be underrepresented (10). Animals can also be sub-clinical carriers of zoonotic pathogens and not show signs of disease therefore, without monitoring, surveillance, and improved diagnostic capacity in animal's zoonotic pathogens may not be identified. Changes in national surveillance case definitions, laboratory testing methods, and policies regarding collection of Indigenous status information may have impacted on the results however, this was outside the scope of this study.

The Indigenous notifications analysed relied on an Indigenous identifier being collected which is commonly underreported (26). Despite original intentions, we were also not able to analyse severity of disease (including health outcomes such as hospitalisations and deaths), or exposure factors due to large amounts of missing data. This limited our understanding of the animal and environmental exposures and subsequent transmission pathways, with evidence of zoonotic transmission between animals and people unconfirmed. Evidence has found that data completeness, timeliness, and inflexibility of the NNDSS database is problematic, with multiple stakeholders at state, territory, and national levels involved in its management (48). Therefore, improved data collection processes that consider the collection of Indigenous identifiers and involve multiple sectors should be considered to improve data completeness, accuracy of analyses, and inform public health responses (43).

4.3. Implications

Implementing integrated systems that involve multiple health sectors could assist with effective management of zoonoses and is an aim of the One Health approach, yet, systems in Australia do not currently facilitate this (33). International examples of joint systems can be seen in relation to antimicrobial resistance, however these systems need further development to truly enact a One Health approach. There is also need to incorporate meaningful inclusion of Indigenous Peoples through developing strong networks and governance structures that promote Indigenous leadership and engagement (39). These collaborative approaches can help to address public health risks at the animal-human-environment interface, improving the prevention and control of zoonoses (49). A One Health Framework can be adopted to prevent and control disease with the collaboration of the animal, human and environmental health sectors likely to be more effective than programmes in a single sector (28). Consistency in the management of zoonoses between health sectors has also been recommended internationally including standardised case definitions and notifiable disease lists for both animals and people, and a coordination centre for reporting and sharing data on zoonotic pathogens and subsequent disease (50). Strengthening communication between sectors including consistency of terminology and training related to zoonoses, and the development of a national One Health plan for addressing zoonoses with shared priorities and responsibilities is also recommended (32, 50).

An existing criterion for communicable diseases to be determined a public health priority and classified as nationally notifiable is the pathogen's importance to Indigenous health (23), therefore a strong evidence base and database is needed to understand the contribution of zoonotic pathogens to human disease (40). This is also needed to undertake a national zoonotic disease prioritisation process which could help to improve the management of zoonoses in Aboriginal and Torres Strait Islander populations. Examples of prioritisation criteria include severity of disease in humans, availability of prevention and control strategies, potential to cause an epidemic or pandemic in

animals or people, and social and economic impacts (50). Whilst some of these criteria may not be relevant for developed countries that have lower prevalence of zoonoses nationally, they are important considerations for Aboriginal and Torres Strait Islander communities that face higher risk of communicable diseases (51).

The relationships and interactions between people, animals, and the environment needs further investigation within Aboriginal and Torres Strait Islander populations, particularly in areas with higher levels of zoonoses notifications. Whilst environmental exposures are integral to the transmission of zoonotic pathogens, environmental health is commonly underrepresented in Indigenous One Health research (29). Environmental health data will continue to be a priority as we see the effects of a changing climate on health outcomes. Australia is considering the development of a national Centre for Disease Control to address emerging and existing health risks and this may address some of the current gaps within the management of zoonoses (52). However, it is yet to be seen if a One Health approach will be supported and how the management of zoonoses within communities will be addressed. Integrated approaches to the management of zoonotic disease and support for Indigenous leadership and governance within the national system is called for to improve the management of zoonoses within Aboriginal and Torres Strait Islander populations.

Futures studies should consider multidisciplinary approaches and further analysis of specific diseases, including trends over time, to improve understanding of zoonoses. Future studies may also include examining the risk of disease related to the social determinants of health (including cultural considerations) (31) and the severity of disease however, this would require holistic data and improved data completeness. Importantly, research within this space should foster Aboriginal and Torres Strait Islander leadership and genuine community engagement, using a transdisciplinary approach, to strengthen partnerships and focus research priorities (53, 54).

4.4. Next steps

This study builds on findings from a zoonoses scoping review that found gaps in the evidence base regarding zoonoses and Aboriginal and Torres Strait Islander populations (40). It also found that despite the strong conceptual foundations of One Health, evidence is lacking in its application and there is a need for research, programmes, and policies that prioritise Aboriginal and Torres Strait Islander leadership, incorporate multiple health sectors, and focus on zoonoses through a One Health approach. These findings will be built on through the development of recommendations for the management of zoonoses in Aboriginal and Torres Strait Islander populations through a One Health approach.

A national integrated One Health system is supported globally and could benefit the management of zoonoses for Aboriginal and Torres Strait Islander populations in Australia. However, consideration of consistency and collaboration between health sectors in the prevention and control of zoonoses for effective management of disease is key. There is also a need for Aboriginal and Torres Strait Islander leadership and engagement in research, policy, and programmes to ensure Australia's zoonotic disease management is effective and appropriate for the population. A continuing challenge is the need for effective partnerships and communication between animal, human and environmental health sectors in research and public health to adopt holistic community health approaches and improve the management of zoonoses.

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.

Author contributions

TR, RL, BC, and JT: conceptualization and methodology. TR and JT: formal analysis and investigation. TR: writing—original draft preparation and project administration. TR, RL, BC, AM, NA, and JT: writing—review and editing. RL, BC, AM, NA, and JT: supervision. 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/fpubh.2023.1175835/full#supplementary-material

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Measuring the impact of an integrated bite case management program on the detection of canine rabies cases in Vietnam

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Introduction: Dog-mediated rabies is enzootic in Vietnam, resulting in at least 70 reported human deaths and 500,000 human rabies exposures annually. In 2016, an integrated bite cases management (IBCM) based surveillance program was developed to improve knowledge of the dog-mediated rabies burden in Phu Tho Province of Vietnam.

Methods: The Vietnam Animal Rabies Surveillance Program (VARSP) was established in four stages: (1) Laboratory development, (2) Training of community One Health workers, (3) Paper-based-reporting (VARSP 1.0), and (4) Electronic case reporting (VARSP 2.0). Investigation and diagnostic data collected from March 2016 to December 2019 were compared with historical records of animal rabies cases dating back to January 2012. A risk analysis was conducted to evaluate the probability of a rabies exposure resulting in death after a dog bite, based on data collected over the course of an IBCM investigation.

Results: Prior to the implementation of VARSP, between 2012 and 2015, there was an average of one rabies investigation per year, resulting in two confirmed and two probable animal rabies cases. During the 46 months that VARSP was operational (2016 – 2019), 1048 animal investigations were conducted, which identified 79 (8%) laboratory-confirmed rabies cases and 233 (22%) clinically-confirmed(probable) cases. VARSP produced a 78-fold increase in annual animal rabies case detection (one cases detected per year pre-VARSP vs 78 cases per year under VARSP). The risk of succumbing to rabies for bite victims of apparently healthy dogs available for home quarantine, was three deaths for every 10,000 untreated exposures.

Discussion: A pilot IBCM model used in Phu Tho Province showed promising results for improving rabies surveillance, with a 26-fold increase in annual case detection after implementation of a One Health model. The risk for a person bitten by an apparently healthy dog to develop rabies in the absence of rabies PEP was very low, which supports the WHO recommendations to delay PEP for this category of bite victims, when trained animal assessors are available and routinely communicate with the medical sector. Recent adoption of an electronic IBCM system is likely to expedite adoption of VARSP 2.0 to other Provinces and improve accuracy of field decisions and data collection.

KEYWORDS
rabies, surveillance system, One Health, Vietnam, dog, veterinary diagnostic

1. Introduction

Rabies is a viral disease that manifests as a progressive, fatal encephalitis. Worldwide, there are an estimated 59,000 human rabies deaths each year, and over 95% are due to dog bites (1, 2). Rabies is entirely preventable if vaccine and immunoglobulin are administered properly and promptly after an exposure. The canine rabies virus variant is enzootic in Asia and approximately 60% of all human rabies deaths occur in this region (1). In Vietnam, rabies control through human and animal vaccination and dog population management in urban areas have been successful. In recent years, 80–100 human rabies deaths are reported annually, compared to more than 400 deaths just a decade ago (3).

In 1996, Vietnam established the National Rabies Control Program to increase support and resources to expand post-exposure prophylaxis (PEP) centers, introduce and revise key legislation and guidelines, and improve One Health collaborations (3). The implementation of this rabies control program resulted in the increase of vaccination centers nationwide, leading to increased PEP availability. Surveillance systems established through the National Rabies Control Program found that over 100 suspected rabid dogs were found in 20 districts across 10 Provinces, from 2008 to 2014 (3). Dog owners have been required to register and vaccinate their dogs since 2017, and data modeling conducted in 2015 estimated the national vaccination coverage in Vietnam to be approximately 43% (3).

In Viet Tri city, Phu Ninh, Lam Thao, and Thanh Ba districts, and Phu Tho town (all located within Phu Tho Province, Vietnam), 15 dog-mediated human rabies deaths were reported from 2010 to 2013 (4). Phu Tho Province has a population of 1.4 million people and is located 100 km northwest of Hanoi. After recognition of the high rate of human rabies deaths in Phu Tho Province by government officials, the provincial Department of Agriculture and Rural Development, in partnership with the World Organization for Animal Health (WOAH) and the Food and Agriculture Organization of the United Nations (FAO), enhanced canine rabies vaccination in the province, increasing coverage from 23% in 2011 to 74% by 2014 (4, 5). Subsequently, reported human rabies deaths in the whole Province of Phu Thu declined to just two human deaths in 2015. Given the success in controlling human rabies deaths, this Province was chosen to pilot an Integrated Bite Case Management (IBCM) program with two goals in mind (1) improve surveillance for animal rabies to inform control measures and (2) to determine if the rabies risk in dogs that have bitten humans is low enough to justify a risk-based approach to providing PEP to dog bite victims.

Vietnam has invested significant resources into controlling rabies to work towards the global goal of zero human deaths from dog-mediated rabies by 2030 (3, 6). Barriers to successful canine rabies control in Vietnam include the lack of accurate dog population estimations, low canine vaccination coverage, lack of rabies awareness among community members, and limited canine rabies surveillance (3). Canine rabies surveillance plays an integral role in rabies control for several key reasons. Surveillance provides epidemiologic data to inform cost-effective control policies and enables monitoring and evaluation of control strategies. Surveillance is also integral for the rapid detection of animal rabies outbreaks. IBCM is a type of rabies surveillance that utilizes a One Health approach to identify suspected rabid animals and human exposures. IBCM has shown to provide additional community benefits, such as removing rabid animals to limit the enzootic rabies transmission cycle (7). IBCM programs also rely on community one health workers (COHWs) who actively seek out bite victims and provide risk assessments and PEP counseling, which can directly reduce human rabies deaths (8). Finally, routine and reliable IBCM programs, where case investigation outcomes are relayed to the patient and medical provider, can reduce the use of unnecessary rabies vaccination when an animal tests negative for the disease (8, 9).

In 2016, an IBCM rabies surveillance program was piloted to improve knowledge of the dog-mediated rabies burden in the Phu Tho Province of Vietnam. This article details the process of developing the program, data of animal rabies cases and bites reported through this program, and a comparison of rabies prevalence and characteristics of data collection methods to the pre-surveillance period.

2. Methods

The Vietnam Animal Rabies Surveillance Program (VARSP) was developed in 2016 under the leadership of Vietnam's Department of Animal Health (DAH) in collaboration with the United States Center for Disease Control and Prevention (US CDC) to strengthen the animal health services of Vietnam capacity to detect, diagnose, and control dog-mediated rabies. VARSP was established in four stages from 2016 to 2019. Historical records for confirmed cases of rabies in Phu Tho, preceding the VARSP program, were available between January 2012 and January 2016.

2.1. Stage one: laboratory development

Prior to 2015, rabies diagnosis was only available within the Vietnam Ministry of Health, which conducted the Direct Fluorescent Antibody (DFA) test and reverse-transcriptase PCR (RTPCR) (10). Diagnostics were primarily conducted on suspected human rabies cases, but animal cases were tested upon request from the DAH. In March 2015, US CDC assisted in the establishment of a national rabies diagnostic facility capable of performing rabies testing using DFA at the National Center for Veterinary Diagnosis (NCVD) in Hanoi. Ten staff were trained on brain tissue removal from deceased animals, sample preparation, reagent management and optimization, and antigen detection by DFA. With financial support from the Global Health Securities Agenda, the laboratory was equipped with one fluorescent microscope, an incubator, freezer, fume hood, and supplies required for processing and diagnosing samples (11). Laboratorians were routinely assessed for DFA proficiency and required to receive rabies pre-exposure vaccination before conducting rabies diagnostic

activities. In 2018 a second laboratory training took place, establishing capacity to perform real time reverse transcriptase PCR.

2.2. Stage two: training of community One Health workers

In August 2015, a two-day training was held for selected COHWs on the principles of rabies surveillance protocols, animal behavior and identifying rabid animals, animal capture and handling, and humane euthanasia for suspect rabid animals. This training included both classroom and live-animal fieldwork components. The eleven participants were current employees of Phu Tho Provincial Sub Department of Animal Health, Health Centers of Phu Ninh and Viet Tri, Veterinary stations of Viet Tri, Phu Ninh and Thanh Ba districts. Eligibility was based on knowledge of the animal health system in Vietnam, previous knowledge of infectious diseases, and ability to clearly communicate information about rabies to bite victims. All COHWs were required to be vaccinated against rabies.

2.3. Stage three: VARSP implementation

Vietnam is comprised of 63 Provinces and centrally-run municipalities which are then divided into districts (12). To establish a framework for rabies detection, control, and elimination, a community-based animal rabies surveillance program was created with a focus on five areas in Phu Tho Province: Phu Ninh (district), Thanh Ba (district), Lam Thao (district), Doan Hung (district) and Viet Tri (capital city Province). The total human population in this area in 2016, based on Phu Tho Province statistics records, is 621,356, with an estimated 109,935 dogs. The above mentioned areas make up a surveillance area that is geographically isolated by a river to the East, a river to the West, and mountains to the North. These geographical barriers make this region of the Phu Tho Province an ideal location to establish a rabies elimination program, as rabies transmission has been shown to be halted or delayed by geographical boundaries such as rivers, mountains, and sparsely populated areas (13). Furthermore, a dog vaccination program supported by WOAH and FAO in this Province was suspected to have significantly reduced dog-mediated rabies virus transmission; however limited surveillance had been conducted to confirm this suspicion (5).

VARSP case investigations were included in this analysis if they occurred between March 2016 and December 2019. Investigations were initiated when COHWs were alerted of a dog bite, suspected rabid animal, or a suspected human rabies exposure from the medical sector or other community cooperators. COHWs met with local community leaders to encourage community-based reporting and established formal information exchange processes with PEP clinics to obtain timely reports of suspected human rabies exposures. Formal information exchange occurred through a network of focal points communicating through telephone call or the social messaging platform Zalo. COHWs or the Provincial Rabies Epidemiologist (PRE) collected information about the location of the animals involved in the bite event and/or animals with symptoms or rabies. A unique patient identification number that links the health record to a bite investigation form was assigned.

VARSP investigations were composed of two parts, community bite investigation and animal investigation, which were typically initiated within 24 h of notification (Figure 1). Animals that had an identifiable owner and appeared healthy were placed on a 10-day in-home quarantine. Animals healthy after 10 days were released from quarantine, and animals that displayed signs consistent with rabies during the investigation or the observation period were euthanized and tested at NCVD. American Veterinary Medical Association standards were used to adopt a local euthanasia protocol (Appendix). Investigation results were reported back to the dog owner, bite victim (s), medical center(s), and other relevant stakeholders so human medical treatment could be adjusted as necessary and appropriate epidemiologic interventions could be implemented. Additional persons identified to have been exposed during the course of the community bite investigations were referred to nearby medical facilities for PEP evaluation.

Data from investigations were documented on paper investigation forms which collected 55 variables including information on the overall health status of the animals, presenting clinical signs, health history, and human exposures (Supplementary material). This information was used for manual determination of the interim and final case status of the animal (non-case, suspect, probable, confirmed), PEP recommendations, and programmatic evaluation. From March 2016 until August 2019 VARSP operated solely as a paper-based collection system that was hand entered into an access database on a monthly basis and shared with Regional, National, and CDC on a quarterly basis.

2.4. Stage four: electronic data capture

In September 2019, IBCM focal points of the Phu Tho Provincial Sub Department of Animal Health (SDAH), district veterinary stations, and staff of Phu Tho provincial Center for Diseases Control (provincial CDC) attended a two-day training on the use of the Rabies Exposure Assessment and Contact Tracing (REACT) App (14). The mobile application is an improvement to paper form data collection as it automates the decision-making process for surveillance officers, allows for more robust data collection, and increases efficiency in real-time reporting. Surveillance data collected is uploaded to a cloud-based server where surveillance data can be viewed and managed by the SDAH leader, DAH, and other approved users. From October 2019 through December 2019, COHWs had the option to use either the REACT electronic system or paper-based data collection. Electronic and paper-based data were merged at the end of the study period in SAS version 9.3.

2.5. Surveillance case definitions

A case definition was developed to assign a case status to animals investigated through VARSP (Box 1).

2.6. Data collection and analysis

Investigation and diagnostic data collected between March 2016 through December 2019 were compared with historical records of

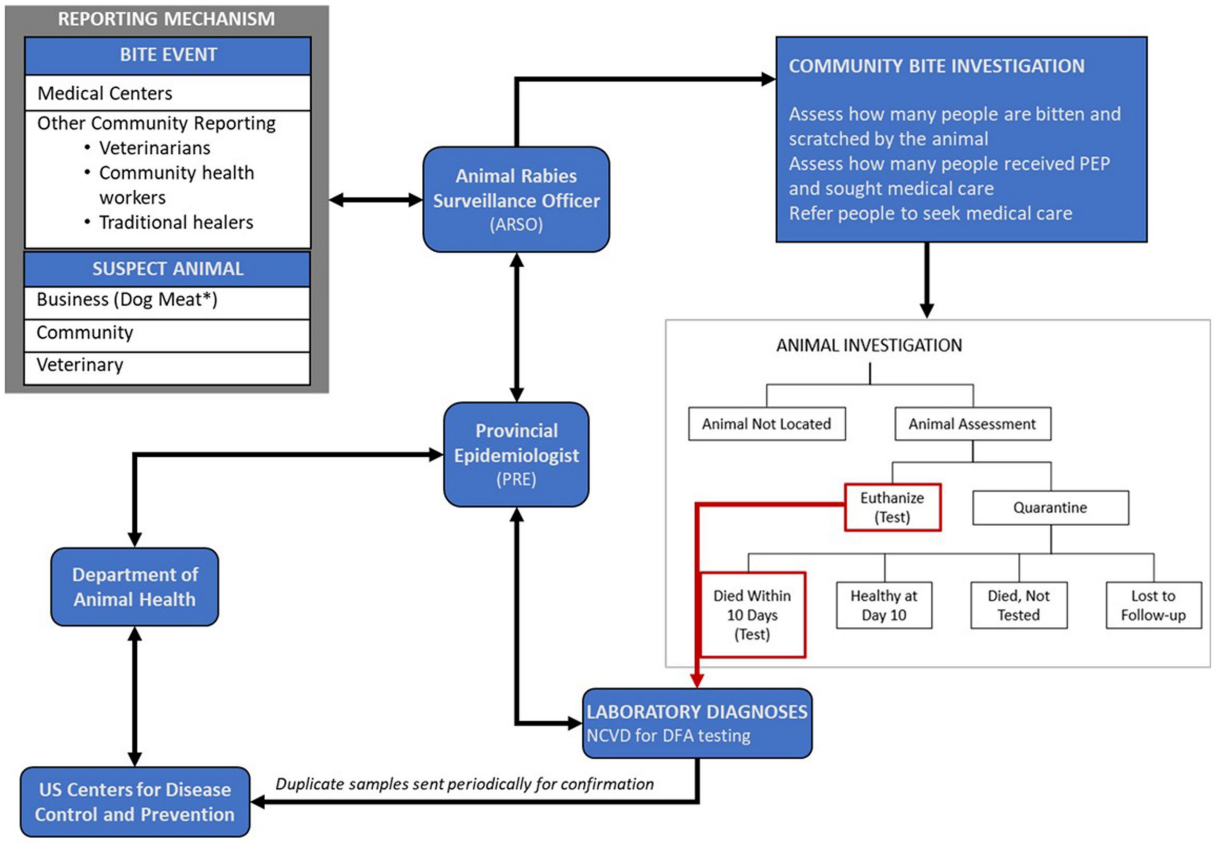

FIGURE 1
Vietnam Animal Rabies Surveillance Program structure for reporting, investigations, diagnostic testing of suspected rabid animals. Arrows represent the flow of information.

BOX 1 Vietnam animal rabies surveillance program case definitions*.

- **Confirmed**
  - If the test results were positive for rabies by DFA or RTPCR
- **Probable**
  - If the animal was not tested for rabies and did not pass quarantine, AND
    - If the animal exposed two or more people or animals and showed 1 or more signs of rabies, OR
    - If the animal showed three or more signs of rabies (hypersalivation, paralysis, lethargy, abnormal vocalization, or unprovoked abnormal aggression), OR
    - Regardless of exposures or symptoms, if the animal died in quarantine, OR
    - If the animal showed one or more signs of rabies and was lost or left during quarantine, OR
    - If the animal showed one or more signs of rabies but was killed or died prior to assessment, OR
    - If the animal was killed or died prior to assessment and exposed two or more people or animals
- **Suspect**
  - A case that is compatible with the WHO clinical case definition of animal rabies or any case reported by a rabies bite center (9)
- **Not a Case**
  - If the test results were negative, OR
  - If the animal was healthy after 10 days in quarantine

*CAVEAT: for subset of observations where investigations were pre-emptively ended
- **Probable**: If animal showed abnormal signs, was reported as not healthy, and exposed 2 or more humans or animals
- **Suspect**: If otherwise

animal rabies cases and dog vaccination data collected by DAH dating back to January 2012. Data were entered into a Microsoft Access database or downloaded from the REACT server as a .csv file and exported to SAS software (version 9.3, SAS Institute Inc., Cary, NC, US). Univariate, descriptive analyses for temporal and spatial trends were conducted. Odds ratios (OR) and conditional maximum likelihood estimate tests of associations between clinical signs and case definitions (i.e., confirmed case, probable case, suspect case, or non-case) were examined to validate the case definition algorithm. A probabilistic risk analysis similar to that described by Medley et al., and published in the WHO Technical Report Series for Rabies, was conducted to characterize the risk of rabies death, assuming no PEP was initiated by exposed persons, under varying rabies exposure scenarios (9, 15, 16). A formal waiver was obtained from the CDC National Center for Emerging Zoonotic Infectious Diseases human subject's advisor; this work was deemed exempt, non-research.

3. Results

During the 46 months of VARSP data included in this analysis, 1,048 investigations were conducted which identified 79 (8%) confirmed rabies cases and 233 (22%) probable cases (Table 1). The majority of investigations conducted on high-risk (confirmed and probable cases) cases were in dogs (95.5%). Other animals investigated included 66 cats, 1 cow, and 4 unnamed animal species. Among animals investigated, 245 were submitted for testing and 79 (32%) were confirmed positive (Figure 2). Of all confirmed cases, 77 (97%) were dogs, 1 (1%) was a cat, 1 (1%) was an unspecified animal species (incomplete data on a paper form). Investigations were reported from multiple sources including human health sectors ($n = 859$), animal health sectors ($n = 117$), and the public ($n = 43$). The majority of confirmed and probable rabid animals were reported from the human health sector (87%).

Prior to the implementation of VARSP, between 2012 and 2015, there was an average of one rabies case investigation per year, resulting in an average of one confirmed or probable rabid animal detected (Figure 3). After the introduction of IBCM, there were, on average, 262 rabies investigations conducted per year (262-fold increase) and 20 laboratory confirmed cases detected (7%) resulting in an over 78-fold increase in annual case detection after implementation. External funding from US CDC supported 796 investigations over the 46-month study period. As a result of the training, and supported by local funding, an additional 300 investigations were conducted by local DAH officials over the same time period. This represents a 28.6% increase in rabies surveillance activities as an indirect benefit of this externally funded program.

VARSP investigations were conducted in 12 of 13 districts in Phu Tho, with rabid animals reported from 8 districts (Figure 4). The six VARSP-focus districts accounted for 91% of investigations conducted and identified 76 rabid animals. Confirmed and probable cases made up 50% ($n = 3$) of investigations in Phu Tho city, 38% ($n = 26$) of investigations in Doan Hung district, 37% ($n = 110$) of investigations in Phu Ninh district, 30% ($n = 64$) of investigations in Viet Tri city, 29% ($n = 35$) of investigations in Thanh Ba district, and 20% ($n = 48$) of investigations in Lam Thao district.

Often-cited signs of rabies (hypersalivation and paralysis) were not commonly recognized among confirmed rabid dogs by the COHWs, at 10 and 5%, respectively (Table 2). Lethargy was commonly noted among rabid animals (32%) but was not significantly associated with animals confirmed as rabid, as it was common among all dogs investigated by COHWs ($p = 0.22$). Odds ratios are significantly higher for most clinical signs in confirmed rabid animals and associations between rabies and these clinical factors had a diminishing relationship with case status (probable, suspect, and non-case). The highest odds of an animal having rabies were observed when they had at least 2 symptoms (OR = 7.8), when they exhibited hypersalivation (OR = 6.1), or when they displayed unexplained aggression (OR = 2.9).

Of all confirmed cases, 52% ($n = 41$) fit the probable clinical case definition and 48% ($n = 38$) fit the suspected clinical case definition. Additional case definition validation occurred through the evaluation of dogs with known outcomes. The probability of a dog, identified by a COHW as meeting the clinical case definition for rabies (probable), being confirmed for rabies through laboratory testing, if such testing were conducted, was 49%. The probability of a dog meeting the suspected rabies case definition being laboratory-confirmed for rabies, if testing were performed, was 8%. This decline in probability further supports the case definitions used in Vietnam.

As part of the bite investigation, COHWs document each bite occurrence. Data collected show a high rate of PEP received regardless of final animal outcome or risk-level of the exposure (Table 3). After a dog bite that was reported to VARSP, 97.2% of people exposed received PEP regardless of case investigation findings. Among all human exposures to dogs, 97.4% of people received PEP. Of those who received PEP, 22.3% were found to have been at low risk for rabies and 36.3% were found to be at no risk for rabies. Of those with no risk for rabies, 96.6% received PEP.

We conducted a probabilistic risk assessment to evaluate the risk of death as a result of being exposed to rabies virus based on data collected over the course of an IBCM investigation (Table 4). Dogs that exposed multiple people ($n = 37$) posed a higher risk for rabies (73%). The exposure with the highest risk of death was a bite to the head or neck by a dog that exposed multiple people (32.8%). For dogs that were healthy and available for quarantine ($n = 147$), the risk for a bite victim to succumb to rabies was low (0.7%) and there is no risk to the bite victim when dogs were healthy after the 10-day quarantine period. The probability of developing rabies was low (<1%) when there were minor bites with no breaks in the skin. If a person was bitten on an extremity by a healthy dog that was available for quarantine, the risk that they would develop rabies in the absence of rabies PEP was 0.03 (three human rabies deaths per 10,000 people under these conditions). Assuming the typical five-dose vaccination series costs $75 USD, this equates to a cost of $220,588.24 USD per death averted, which is 26x higher than the WHO cost-effectiveness indicator of 3-times the GDP *per capita* (17). In comparison, a person who was bitten on an extremity by a dog exhibiting at least one clinical sign of rabies, the cost per death adverted is $7,500 USD, which is below the WHO cost effectiveness indicator.

4. Discussion

4.1. Establishment of routine surveillance

The rabies surveillance system implemented in Phu Tho Province, Vietnam is based on the World Health Organization

10.3389/fpubh.2023.1150228

TABLE 1 Rabies case status by animal species, Phu Tho Province, Vietnam March 2016 – December 2019.

Case definition	Confirmed *N* (%)	Probable *N* (%)	Suspect *N* (%)	Non-Case *N* (%)	Total
Dog	77 (8%)	221 (23%)	230 (24%)	449 (46%)	977
Cat	1 (2%)	11 (17%)	24 (36%)	30 (45%)	66
Other	1 (20%)	1 (20%)	2 (40%)	1 (20%)	5
Total	**79 (8%)**	**233 (22%)**	**256 (25%)**	**480 (46%)**	**1,048**

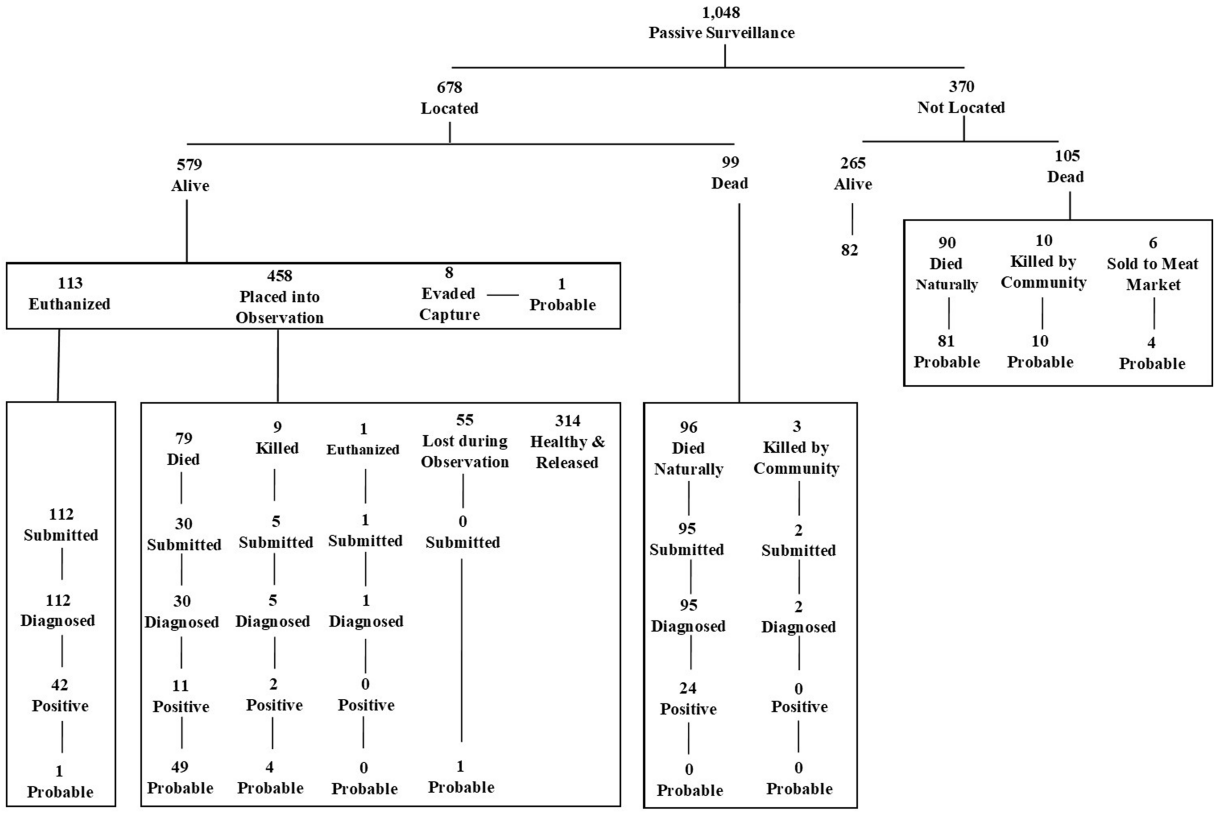

FIGURE 2
Outcomes of investigations and diagnoses of animals investigated through the Vietnam Animal Rabies Surveillance Program, Phu Tho Province, March 2016 – December 2019.

(WHO)-supported IBCM approach for investigation and data recording that has also been implemented previously in dog-mediated rabies endemic countries (18, 19). IBCM integrates data collection and reporting at the community level of animal and human health sectors to form a cohesive response to managing animal bites and PEP recommendations. Outcomes of this One Health approach can lead to reduced rabies transmissions in communities through rapid response, animal evaluation, contact tracing, and implementation of control measures (18, 19). Accomplishing a functional IBCM program in Vietnam entailed the training of health care and public health workers, establishing laboratory capacity, raising community awareness of rabies, and development of systems and protocols to ensure that investigation results involving animal bite victims were shared with PEP providers in a timely manner. As a result of this One Health approach, fostering collaboration between the human health sector and the animal health sector, COHWs were able to investigate 859 bite cases which made up the majority of the investigations conducted in the VARSP-focused districts. This highlights the importance of multi-sector communication to bolster animal rabies surveillance systems. Key outcomes of these efforts were two-fold: (1) to reduce the rabies burden and human lives lost, and (2) more effective and efficient use of human and dog vaccines.

The establishment of a case definition is a foundational component of any surveillance system as it standardizes the criteria to identify cases and defines when public health and animal health interventions are indicated. Animals that test positive fall into a standard "confirmed" case definition and animals that test negative or pass the 10-day quarantine period fall into a standard "non-case" definition. However, many animals investigated for bites in rabies endemic countries have no definitively known final health outcome, often due to low rates of animal ownership and proclivity for owned dogs to roam freely among other factors. Animals that are not available for testing or quarantine, or that test inconclusively, are often harder to classify and fall into "probable" or "suspect" case definitions. These

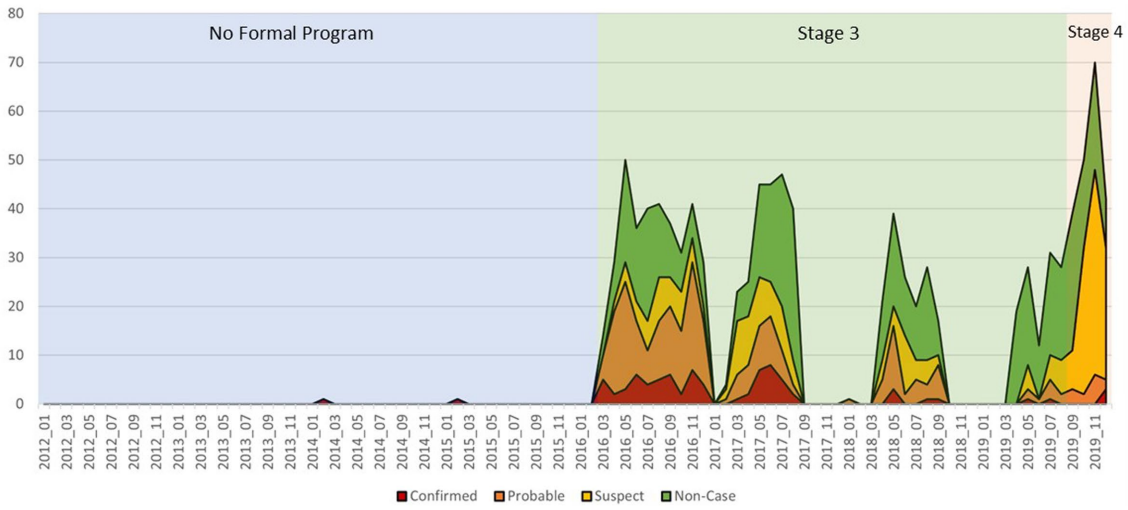

FIGURE 3
Animal rabies case statuses by date of diagnosis, Phu Tho, January 2012–December 2019.

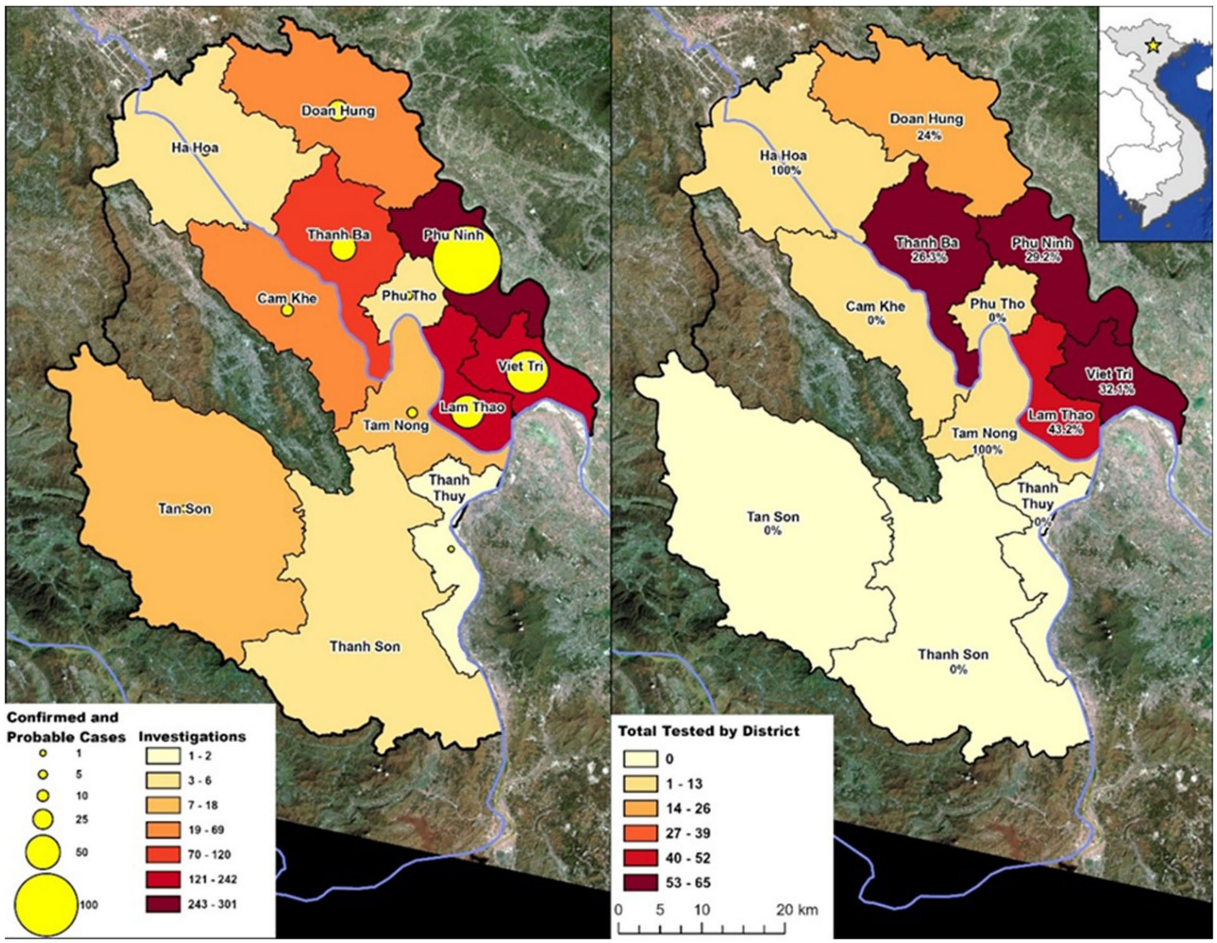

FIGURE 4
Animal rabies activities in Phu Tho Province by district, March 2016–December 2019. Percent positive of tested samples are indicated below district name. Data retrieved from usgs.gov. Landsat 8–9 OLI/TIRS C2 L2 (09-30-2019).

definitions have multiple levels of considerations and have been shown to be inconsistently applied across rabies programs, particularly when depending upon paper-based or non-standardized information collection systems (8). Odds ratios among rabid animals in VARSP were significantly higher for clinical signs such as aggression, biting, and hypersalivation, but these associations were progressively reduced

TABLE 2 Clinical signs observed during investigation of suspected rabid animals by case status, Phu Tho Province, March 2016 – December 2019.

	Confirmed			Probable			Suspect			Non-case		
Clinical signs	*N*	%	OR	*N*	%	OR	*N*	%	OR	*N*	%	OR
Aggression	52	66%	2.9**	123	53%	2.3**	55	21%	0.9	111	23%	
Biting	35	44%	1.8**	79	34%	1.4*	47	18%	0.7	119	25%	
Hypersalivation	8	10%	6.1**	6	3%	1.6	1	0%	0.2	8	2%	
Paralyzation	4	5%	3.0	7	3%	1.8	0	0%	–	8	2%	
Lethargy	25	32%	1.4	94	41%	1.7**	5	2%	0.1**	112	23%	Ref
Total	**79**	**100%**		**232**	**100%**		**257**	**100%**		**481**	**100%**	
0	13	16%	0.3**	49	21%	0.4**	192	75%	1.4**	259	54%	
1	43	54%	1.3	136	59%	1.4*	62	24%	0.6**	203	42%	
2	23	29%	7.8**	47	20%	5.4**	3	1%	0.3	18	4%	

*Indicates significance at 0.05. **Indicates significance at 0.01.

TABLE 3 Human exposures to suspected rabid animals by case status and species, Phu Tho Province, March 2016 – December 2019.

Animal	Case definition	Bites				Scratches/other				Total persons exposed			
		People bitten		People received PEP		People scratched		People received PEP		Total exposures		Received PEP	
		N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
Dog	Confirmed	107	11.1%	103	96.3%	1	7.7%	1	100.0%	108	11.1%	104	96.3%
	Probable	291	30.2%	290	99.7%	5	38.5%	5	100.0%	296	30.3%	295	99.7%
	Suspect	215	22.3%	207	96.3%	3	23.1%	3	100.0%	218	22.3%	210	96.3%
	Non-case	350	36.3%	338	96.6%	4	30.8%	4	100.0%	354	36.3%	342	96.6%
Total		**963**	**100.0%**	**938**	**97.4%**	**13**	**100.0%**	**13**	**100%**	**976**	**100.0%**	**951**	**97.4%**
Other	Confirmed	2	2.8%	2	100.0%	0	0.0%	0	0.0%	2	2.6%	2	100.0%
	Probable	14	19.7%	14	100.0%	3	60.0%	3	100.0%	17	22.4%	17	100.0%
	Suspect	25	35.2%	23	92.0%	1	20.0%	1	100.0%	26	34.2%	24	92.3%
	Negative	30	42.3%	28	93.3%	1	20.0%	1	100.0%	31	40.8%	29	93.5%
	Total	**71**	**100.0%**	**67**	**94.4%**	**5**	**100.0%**	**5**	**100.0%**	**76**	**100.0%**	**72**	**94.7%**
Total	Confirmed	109	10.5%	105	96.3%	1	5.6%	1	100.0%	110	10.5%	106	96.4%
	Probable	305	29.5%	304	99.7%	8	44.4%	8	100.0%	313	29.8%	312	99.7%
	Suspect	240	23.2%	230	95.8%	4	22.2%	4	100.0%	244	23.2%	234	95.9%
	Negative	380	36.8%	366	96.3%	5	27.8%	5	100.0%	385	36.6%	371	96.4%
	Total	**1,034**	**100.0%**	**1,005**	**97.2%**	**18**	**100.0%**	**18**	**100.0%**	**1,052**	**100.0%**	**1,023**	**97.2%**

for lower-risk case status (probable, suspect, non-case). This decrease in associations supports the case definitions used for probable and suspect rabid animals by VARSP.

The surveillance system implemented in Vietnam is based on the same model that was adopted in Haiti, with slightly different case definitions reflecting input from the National Rabies Program. As a result, a higher proportion of investigations in Vietnam resulted in a probable case definition when compared to the results in Haiti (22% vs. 4.6%). These differences can be attributed to the different case definitions used in each country. The probable case definition used in Vietnam covers a broad scope of scenarios to account for the lack of availability of the animal for testing, clinical signs of rabies during the assessment, quarantine result, and the number of human and animal exposures for probable cases. Haiti's probable case definition differs in that the number of human and animal exposures are not included in the algorithm to define a probable case (9, 18). Among animals that could be tested, Vietnam's percent positivity among dogs meeting the probable case definition was consistent with the results observed in Haiti (49% vs. 50%) which may suggest that among animals eligible for testing and under the structure of this IBCM approach, there is a consistent likelihood of detecting rabies virus infections. The variation in case definitions between the two countries which followed the same IBCM model highlights the importance of designing a case definition that supports the specific program, but also showcases how important case definitions are when analyzing and interpreting surveillance data.

While some variables are highly associated with rabies case status in biting dogs, no single variable alone was adequate for the basis of a risk assessment and determining if PEP is indicated (15, 20). Rabies cases were documented among dogs with a reported history of vaccination, among owned dogs, and among dogs originally considered low risk at the time of assessment. As seen in other studies, a combination of risk factors must be considered to make an accurate

TABLE 4 Probability of succumbing to rabies virus infection after exposure to dogs in Phu Tho Province, 2016–2019.

Exposure consideration	Probability of death based on level of exposure	Information collected at time of exposure							Quarantine or testing	
		Dog exposed multiple people	Dog dead within 10 days of exposure	Unowned Dog	Dog bite was not provoked (Aggressive)	Dog not vaccinated	Dog is showing >1 clinical sign of rabies	Dog healthy and available for quarantine	Dog healthy at 10 days post-bite	Tested negative
Bite(s) to head/neck	45%	32.8%	25.3%	20.5%	15.1%	10.7%	9.4%	0.3%	0.0%	0.0%
Multiple severe bite wounds	27.50%	20.1%	15.5%	12.5%	9.2%	6.6%	5.7%	0.2%	0.0%	0.0%
Bites to young children	27.50%	20.1%	15.5%	12.5%	9.2%	6.6%	5.7%	0.2%	0.0%	0.0%
Bites to extremities	5.00%	3.6%	2.8%	2.3%	1.7%	1.2%	1.0%	0.0%	0.0%	0.0%
Minor bites (no break in skin)	1.00%	0.7%	0.6%	0.5%	0.3%	0.2%	0.2%	0.0%	0.0%	0.0%
Probability the dog has rabies		73.0%	56.3%	45.5%	33.6%	23.9%	20.8%	0.7%	0.0%	0.0%
Confirmed		27	27	5	45	63	59	1	0	0
Eligible dogs (n)		37	48	11	134	264	283	147	285	61

Probabilities are shaded on a scale from green, yellow, orange, red to represent the range of risk with green representing the lowest risk and red representing the highest risk.

assessment of the rabies status of a biting dog (15, 19). In this analysis, biting dogs that are assessed by an animal health professional to be healthy and are available for in-home quarantine by their owners were highly unlikely to develop rabies and represent a very low risk of rabies transmission to their bite victims (0.03%). These findings are similar to those in Haiti where bites from dogs that were assessed as healthy and available for quarantine represented a 0.05% risk of rabies to the bite victim and further supports the WHO IBCM protocol (15). WHO recommends that PEP be provided based upon an appropriate risk assessment by a health professional, where available (9). The results of this evaluation show that in the Phu Tho Province, the animal health professional's assessment of the animals was accurate and when combined with the ability to self-quarantine a healthy dog, the risk of delaying PEP for the majority of bite victims is negligible. This analysis also highlights that treating all persons with PEP for these low-risk exposures is not cost-effective. At least two countries have shown that a protocol in which persons delayed PEP while healthy dogs were under quarantine would present almost no risk to the bite victim, as they would receive PEP in the rare situation if the dog developed rabies during the 10-day quarantine period (15, 19).

4.2. Rabies burden

During the study period, VARSP was able to identify 79 laboratory confirmed rabid animals. This is a dramatic increase compared to 2012–2015 where only two confirmed cases were reported. There are three time periods with no or very few investigations (Figure 3). This should not be interpreted as a decline in suspect rabid dogs during these time periods, but rather an indicator of the funding mechanisms not being available and surveillance efforts temporarily halted. Also during this time was a decline in the proportion of high-risk cases (6-fold decrease in proportion of high-risk cases from 2016 to 2019/51.6 vs. 8.5%). During 2016–2017, 40 high-risk dogs were euthanized and removed from the community, and this may have limited the spread of rabies in the community. However, this impact is likely to be small and does not fully account for the decline in high-risk cases which were observed. Dog vaccination efforts declined in 2016, after cessation of a WOAH and FAO-supported dog vaccination program which provided free vaccines to dog owners (5). During the study period, routine vaccination campaigns were held by DAH, but dog owners were charged approximately $1 per rabies vaccine and vaccination coverages by this approach were thought to reach only 40% of the dog population. The cause of the apparent reduction in high-risk rabies cases in Phu Tho during the study period is unknown but could reflect enzootic multi-year cycles of disease transmission (21–23).

4.3. Benefits of routine animal rabies surveillance

The IBCM system implemented in Vietnam is a WHO-supported approach to rabies surveillance that integrates data collection and reporting at the community level with animal and human health sectors to form a cohesive response to animal bites. It accomplishes this by limiting rabies transmission in communities through rapid response, animal evaluation, contact tracing, and implementation of control measures; this entails the training of health care and public

health workers, raising community awareness of rabies, and referring animal bite victims to existing centers where post-exposure prophylaxis (PEP) can be received.

In March 2018, an official decision on the implementation of Event Based Surveillance (EBS) in Vietnam was issued (24). Accordingly, dog bites became a signal required to be reported to the health system for early warning and response. In addition, animals (especially dogs) with signs consistent with rabies are required to be notified to local human health or animal health authorities. If implemented properly, EBS will enhance the coordination and more frequent information exchange between the animal and human health sectors, improving the prospects for expanded implementation of IBCM, coordinated rabies surveillance and outbreak response efforts, following a One Health approach.

Vietnam is one of only a few rabies endemic countries in Southeast Asia where PEP is widely available. The majority of medical centers follow the Essen 5 dose schedule while others deliver vaccine using the Updated Thai Red Cross 8-dose schedule (25). Using the surveillance data collected over the course of the VARSP operation, PEP could have been averted for 366 people (92 people per year) and safely delayed for 150 people who were exposed to healthy dogs available to be quarantine. However, PEP was still regularly administered under this pilot program as WHO does not recommend risk-based PEP unless there is a functional surveillance system in place to ensure appropriate risk assessment and animal follow up is conducted. PEP in Vietnam costs $75 per person for the complete series. This represents a potential PEP savings among the study population of $6,900. Future IBCM program implementation should consider using investigation results to inform PEP decisions in low-risk situations where PEP can safely be delayed. The cost-savings in PEP could be used to supplement IBCM operational costs or complementary dog vaccination programs (26).

The transition of Vietnam's Animal Rabies Surveillance Program from a paper-based system to an app-based system (REACT) offers several advantages. Due to the complex nature of rabies investigations, using an app-based approach removes some of the time-sensitive decisions that COHWs must make pertaining to the risk of the animal having rabies and the appropriate PEP recommendation for exposed individuals and potential euthanasia decisions for animals. Interviews with surveillance officers in Haiti who used both paper-based and app-based systems determined that the majority of surveillance officers agreed with statements pertaining to the ease of use, timeliness of report submission, rabies risk assessment, and timeliness of data analysis (14). In addition, this system allows for more detailed data collection that would not otherwise be documented in a paper-based system. Lastly, the use of an app-based system allows for more timely and impactful data sharing with local partners, national programs, and international collaborators. Having access to near real-time surveillance allows for more efficient management of rabies surveillance in Vietnam and provides a strong impetus to continue funding rabies prevention activities. Since 2020, DAH has trained an additional 369 COHWs on the VARSP and REACT module for expanded rabies surveillance.

4.4. Limitations

As seen in similar IBCM implementation scenarios, the passive surveillance system in the Phu Tho Province relies on notifications from medical centers and the community to initiate data collection. The human population of the study area in 2016 was 621,356. Estimates by Hampson et al. estimate the annual bite rate in Vietnam as 378/100,000 (1). By these estimates, we should expect to see approximately 2,349 bites meaning approximately 88% of bites are not being reported or investigated. This is likely due to multiple factors, including the lack of healthcare seeking, periodicity in VARSP operations, and missed opportunities for case notifications. The impact of under-detection of bite cases on our knowledge of rabies epidemiology in Pho Tho is unknown. In addition, the system is designed to investigate dogs, the primary rabies reservoir in Vietnam. As a result, very few spillover species were investigated under this protocol, resulting in poor characterization of non-dog rabies transmission in the program area.

5. Conclusion

The pilot IBCM model used in Phu Tho Province showed promising results for being a cost-effective strategy to improve rabies surveillance, with a >78-fold increase in annual case detection after implementation. The risk for a person bitten by an apparently healthy dog to develop rabies in the absence of rabies PEP was very low, which supports WHO guidance to delay PEP for this category of bite victims, when trained animal assessors are available to evaluate the offending dog. While WHO recommends a risk-based approach under the aforementioned conditions, the ultimate decision to pursue PEP should be made between the practitioner and the patient. The recent adoption of an electronic IBCM system is likely to expedite adoption of this IBCM program to other Provinces to further improve rabies surveillance and resulting health outcomes nation-wide.

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 approved by Institutional Animal Care and Use Committee, US Centers for Disease Control and Prevention. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CV, MP, DN, TXN, TTN, LO, TDN, ON, TD, AD, and LN contributed to conception and design of the study. RW and RDT (REACT Development Team) organized the database. YR, CV, SB, and RW performed the statistical analysis. YR wrote the first draft of the manuscript. YR, CV, LO, RW, and RDT (REACT Development Team) wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

REACT Development Team was lead for the development of the electronic application as part of employment for Mission Rabies.

The remaining 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/fpubh.2023.1150228/full#supplementary-material

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A follow-up study of 100 patients with acute brucellosis for its prognosis and prevention

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Objective: To prevent chronic brucellosis, this study analysed the changes in patient antibody titers, and the trajectories of biochemical indicators at different stages of brucellosis, identified relevant biomarkers, and explored risk factors affecting the prognosis of brucellosis patients.

Methods: A prospective cohort study was conducted to follow 100 patients with acute brucellosis. Laboratory serological test results [taken with a serum (tube) agglutination test (SAT)] and biochemical parameters (liver function, renal function, and hematological system) were measured repeatedly at four-time points: 0 weeks—baseline survey, 6 weeks after the first treatment, 12 weeks after the second treatment, and 3 months after the third treatment. The changes in the antibody titres and biochemical parameters at each time point were analysed for trend changes.

Results: One hundred patients with acute brucellosis were enrolled in this follow-up study, with 100% retention in follow-up. By the third follow-up, 21 patients had turned subacute and 11 had turned chronic. One-way repeated measures analysis of variance results showed statistically significant differences ($p < 0.01$) across the time points for the following five indicators: alanine aminotransferase, aspartate aminotransferase, total bilirubin, serum creatinine (SCr) and platelet count. The clinical symptoms of patients in the acute stage were mainly joint pain, fatigue, and fever, while those in the chronic stage complained primarily of joint pain and fatigue. The results of multivariate logistic analysis showed that joint pain [odds ratio (OR) = 3.652, 95% confidence interval (CI) =1.379–9.672], monoarticular pain (OR = 6.356, 95% CI = 4.660–8.669), elevated SCr (OR = 15.804, 95% CI = 1.644–151.966) and elevated haemoglobin (Hb) (OR = 1.219, 95% CI = 1.065–1.736) were risk factors for poor prognosis (not cured or chronic) in patients with brucellosis.

Conclusion: The trajectory of changes in patient SAT posirates and antibody titers can be used to distinguish patients with chronic brucellosis. The brucellosis is preventable and treatable, and the standard treatment can be effective in reducing the clinical symptoms of affected patients. If patients are not treated in a timely manner, joint pain, monoarticular pain, and elevated SCr are risk factors for patients who are not cured. Therefore, the treatment cycle for these patients should be extended.

KEYWORDS

brucellosis, case follow-up, repeated measures, antibody titres, biochemical parameters, clinical symptoms

Highlights

- Here is what is already known on this topic:

1. Brucellosis is a zoonotic allergic disease caused by bacteria of the genus *Brucella* and has become an important public health problem worldwide.
2. Common symptoms after *Brucella* infection in humans include fever, fatigue, loss of appetite and joint pain.
3. Chronic brucellosis has atypical clinical manifestations that are often recurrent and clinically difficult to cure.

- Here is what this study adds:

1. The early diagnosis and standard treatment of patients with acute brucellosis can effectively control the disease.
2. The serum (tube) agglutination test positive rate and antibody titre gradually decrease over the course of the acute phase of brucellosis.
3. After receiving uniform standard treatment, patients' symptoms (e.g., fever, fatigue, hyperhidrosis and polyarticular pain) decreased continuously.
4. Joint pain and single joint pain are risk factors for patients' failure to be cured.

- Here is how this study might affect research, practice or policy:

1. There is a certain trajectory between the course of acute brucellosis and the serum (tube) agglutination test positive rate, antibody titre and alanine aminotransferase level.
2. Patients in the acute phase of brucellosis can effectively relieve and eliminate their clinical symptoms through a full course of standardised treatment.
3. The treatment cycle should be extended for patients who develop certain symptoms such as joint pain.

1. Introduction

Brucellosis is a zoonotic disease caused by bacteria of the genus *Brucella* (1) and a typical natural epidemic infectious disease. Common symptoms of brucellosis in humans include fever, malaise, loss of appetite, and joint pain, which in turn may lead to complications such as meningitis and arthritis, affecting the patient's ability to work and possibly leading to death in severe cases (2). Human-to-human transmission of *Brucella* is rare, while some livestock-related human infections are caused by contact with infected animals such as sheep and cattle, especially during the delivery of lambs (3). *Brucella* can spread via airborne mechanisms in certain circumstances (4, 5). At present, the epidemic situation of brucellosis is seriously underestimated and has become an important global public health concern (6, 7); additionally, the World Health Organization believes that brucellosis is one of the most easily overlooked zoonoses (8).

The clinical symptoms of brucellosis in the acute phase are typical, and can be cured with a full course of timely standardised treatment. Chronic brucellosis, which has atypical clinical manifestations is difficult to cure clinically. Patients with chronic brucellosis often have recurrent clinical manifestations; some patients have serious sequelae, while others even lose their ability to work. Due to the lack of specialised treatment for chronic brucellosis, its repeated symptoms, and its prolonged disease course, patients bear a heavy economic burden due to the medical treatment costs. In addition, the infected animals must be treated, adding an even heavier financial burden to patients with chronic brucellosis. At present, there is no exact laboratory differential test for chronic brucellosis, which is mainly determined according to the duration of the disease; the course of the disease in many patients is mainly determined by the patients' chief complaints (9), resulting in different disease stages in one patient being determined by various doctors. These issues directly affect the standardised treatment and prognosis of patients with brucellosis, and they even affect the accurate implementation of overall prevention and control in a region. It is reported that approximately 5%–15% of patients with brucellosis in China still change from acute to chronic every year (10). This study analyses whether there is a trajectory in the changes of antibody titres and biochemical parameters during each course of the disease to provide a theoretical basis for finding markers associated with chronic brucellosis. At the same time, through analyzing the relevant contents of the questionnaire, this study determines the risk factors that affect the prognosis of patients with brucellosis and provides a scientific basis for preventing the disease from turning chronic, thus improving the survival quality of patients.

2. Materials and methods

2.1. Study subjects

This was a prospective study. This study selected the Disease Control and Prevention Centre of Wuchuan County, Inner Mongolia Autonomous Region, as the research site, and it included patients with acute brucellosis according to the Diagnostic Criteria for Brucellosis (11). The confirmed patients were treated in designated medical institutions and completed the study's baseline questionnaire after giving informed consent.

2.2. Inclusion criteria

Patients with brucellosis were included using the Diagnostic Criteria for Brucellosis WS269-2019 (11). They were aged 18–70 years, and the study's survey was administered according to the principle of informed consent until the sample size was met. According to the Diagnostic Criteria for Brucellosis WS269-2019, patients were included who had an epidemiological history and clinical manifestations of the disease and met any of the following conditions: ① the titre of the serum (tube) agglutination test (SAT) was 1:100 or above; ② the titre of the anti-human immunoglobulin test was more than 1:400; and ③ *Brucella* was isolated. The subacute stage of brucellosis was defined if the patient had clinical symptoms related to brucellosis, the course of the disease was within 3–6 months and the laboratory confirmed a serological positive reaction. The chronic stage of brucellosis was defined if the disease lasted more than 6 months, was not cured and had signs related to brucellosis with a serological positive reaction confirmed by the laboratory.

2.3. Exclusion criteria

Patients who met any of the following criteria were excluded: ① those infected with human immunodeficiency virus, undergoing chemotherapy, suffering from other immune system diseases (such as lupus erythematosus) or combined with other serious diseases (such as liver failure, severe hepatitis, liver cirrhosis, renal failure, uraemia, proteinuria, multi-drug resistant pulmonary tuberculosis, severe coronary heart disease, chronic obstructive pulmonary disease, and cerebral infarction); ② patients with mental disorders, deafness, and other diseases resulting in poor communication and unable to cooperate with the survey; ③ pregnant women; ④ patients who did not agree to participate in this study; and ④ patients who were allergic to the therapeutic drugs in this study.

2.4. Sample size

Based on previous literature (12) and a 1 year treatment follow-up period, it was assumed that the chronicity rate of brucellosis was approximately 12%. In addition, the test validity was set at Power = 0.90, the test level at $\alpha = 0.05$, and the sample size $N = 85$ was calculated by SPSS software. Considering possible dropouts, 100 patients with brucellosis were included in this study. Here is the sample size calculation formula:

$$n = Z_{\alpha}^{2} P(1-P) / \delta^{2}$$

P is the chronicity rate and δ is the allowable error.

3. Methods

3.1. Treatment plan

All included patients were treated in strict accordance with the Diagnostic Criteria for Brucellosis WS269-2019 (11). The patients were treated with doxycycline (200 mg/d, twice a day) and rifampicin (15 mg/kg, once a day) in accordance with the principles of early, combined and sufficient treatment.

3.2. Data collection

Standardised questionnaires were used to collect patient information, including general demographic (age, sex, occupation) and epidemiological data (frequency and mode of exposure to animals). Baseline and follow-up surveys and relevant laboratory tests were performed on all patients with brucellosis who met the study's inclusion criteria. The study was approved by the Ethics Committee of the Centre for Disease Control and Prevention of Inner Mongolia Autonomous Region (approval number: 2022011601).

3.3. Baseline survey

The patients were diagnosed according to the Diagnostic Criteria for Brucellosis WS269-2019 (11), and the study subjects were selected according to the inclusion and exclusion criteria. Informed consent forms were signed by the patients included in the study, and the baseline questionnaires were administered to them for uniform and standardised treatment. Blood was collected from the included patients for the SAT, liver function, kidney function, haematological system and other indexes.

3.4. Follow-up investigation

A total of three follow-up surveys were conducted on the included patients, and the follow-up times were 6 weeks after treatment, 12 weeks after treatment, and 3 months after treatment. In addition to the surveys, the SAT, liver function, kidney function, haematological system and other indexes were collected at each follow-up visit.

3.5. Laboratory testing

3.5.1. SAT

Unknown patient serum was added to a suspension of *Brucella* antigens, and if *Brucella* antibodies were present in the patient's serum, then antigens and antibodies reacted specifically to form agglutinates that were visible to the naked eye. The tested serum dilutions were 1:50, 1:100, 1:200 and 1:400. The titre was determined by the highest serum dilution that produced 50% (++) agglutination. Haemagglutination above (++) was considered positive in serum diluted to 1:100 and suspicious in serum diluted to 1:50.

3.5.2. Liver function test

According to the instructions of the kit (manufacturer: Jiangsu Maiyuan Biotechnology Co., Ltd.), a colorimetric determination was performed, the standard curve at a wavelength of 600 nm was prepared, and the alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL) contents were calculated on a standard curve after the blood sample was measured.

3.5.3. Renal function test

Blood urea nitrogen (BUN) and serum creatinine (SCr) were determined by a semi-automatic biochemical analyser (manufacturer: Mindray; model: BA-88A) in the patient's serum.

3.5.4. Haematological test

ALT, AST, TBIL, BUN, SCr, red blood cell (RBC) count, white blood cell (WBC) count, haemoglobin (Hb), platelet (PLT) count and other indicators were measured on an automatic haemocytometer (manufacturer: Mindray; model: BC-2600).

3.6. Analytical and statistical methods

EPIDATA 3.0 was used to establish a database, and the double entry method was used for questionnaire and experimental data entry. The descriptive analysis and statistical inference were performed using SPSS software version 23.0 (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.), and descriptive statistics were reported as absolute numbers and constituent ratios (n, %). Pearson's chi-square test and a two-sided

Fisher's exact test were used to compare enumeration data between two or more groups, respectively, and $p < 0.05$ was considered statistically significant.

One-way repeated measures analysis of variance (ANOVA) was used for biochemical indicators at different survey time points, and a homogeneity test and sphericity test were performed for the data. If $p > 0.05$ for the sphericity test, the data conformed to the sphericity hypothesis, and there was no correlation between the data, so the repeated measures ANOVA was used. If $p < 0.05$, the data did not conform to the sphericity hypothesis, and the Greenhouse–Geisler correction was used.

Potential factors influencing the prognosis of brucellosis were analysed by a univariate logistic regression analysis, and variables that were significant in the univariate analysis ($p < 0.05$) or variables that were considered likely to affect the treatment outcomes were included in the multivariate logistic regression analysis. The criteria for the assignment are listed in Table 1.

TABLE 1 Variable assignment table for logistic regression analysis.

Independent variable	Index category	Assignment criteria
Gender	Male	1
	Female	2
Age	≥45 years	1
	<45 years	2
Asthenia	Yes	1
	No	2
Arthralgia	Yes	1
	No	2
Shoulder pain	Yes	1
	No	2
Knee pain	Yes	1
	No	2
Monoarticular pain	Yes	1
	No	2
Polyarticular pain	Yes	1
	No	2
Joint migration pain	Yes	1
	No	2
On-time medication	Yes	1
	No	2
Alanine aminotransferase increased	Yes	1
	No	2
Aspartate aminotransferase increased	Yes	1
	No	2
Bilirubin increased	Yes	1
	No	2
Blood urea nitrogen increased	Yes	1
	No	2
Creatinine increased	Yes	1
	No	2
Red blood cell increased	Yes	1
	No	2
White blood cell decreased	Yes	1
	No	2
Hemoglobin increased	Yes	1
	No	2
Platelet decreased	Yes	1
	No	2

4. Results

4.1. Overview of included cases

According to the case diagnostic screening rules, a total of 100 patients with brucellosis were included in the follow-up study, with 100% retention in follow-up. Out of the total, 61 patients were males, and 39 were females, with a male-to-female ratio of 1.56:1 and a mean age of 53.81 ± 0.99 years. Their occupations were dominated by farmers; 92 patients were farmers, 5 patients were catering industry practitioners, 2 patients were herdsmen, and 1 patient was a worker. At the baseline survey, 98 patients had been exposed to animals within the past month, including 78 dogs (79.59%), 72 sheep (73.47%), 69 pigs (70.41%), 48 cattle (48.98%), and 2 others (2.04%); 97 patients (98.98%) had been exposed to sheep, cattle, dogs, and pigs. By the third follow-up, 32 patients had positive SAT results and associated clinical symptoms. Over the course of this study, 21 patients turned subacute, 11 patients turned chronic, and the chronic rate was 11%.

4.2. SAT test results

The baseline SAT test was positive in 100 patients, the SAT antibody titre was ≥1:400^{++} in 4 patients, the antibody titre was ≥1:200^{++} in 89 patients and the antibody titre was ≥1:100^{++} in 7 patients.

At the first follow-up visit, there were 82 SAT-positive patients without SAT antibody titres ≥1:400^{++}, 76 (92.68%, 76/82) patients with antibody titres ≥1:200^{++} and 6 (7.32%, 6/82) patients with antibody titres ≥1:100^{++}.

At the second follow-up visit, there were 58 SAT-positive patients without SAT antibody titres ≥1:400^{++}, 50 (86.21%, 50/58) patients with antibody titres ≥1:200^{++} and 8 (13.79%, 8/58) patients with antibody titres ≥1:100^{++}.

At the third follow-up visit, there were 32 SAT-positive patients without SAT antibody titres ≥1:400^{++}, 18 (56.25%, 18/32) patients with antibody titres ≥1:200^{++} and 14 (43.75%, 14/32) patients with antibody titres ≥1:100^{++}.

The trend of antibody titre composition and the positive rate at each survey time point is shown in Figure 1.

The positive rates of the four survey time nodes gradually decreased, and the difference was statistically significant ($p < 0.05$), as is shown in Table 2. In different disease courses, the proportion of patients with an antibody titre ≥1:200^{++} accounted for the highest

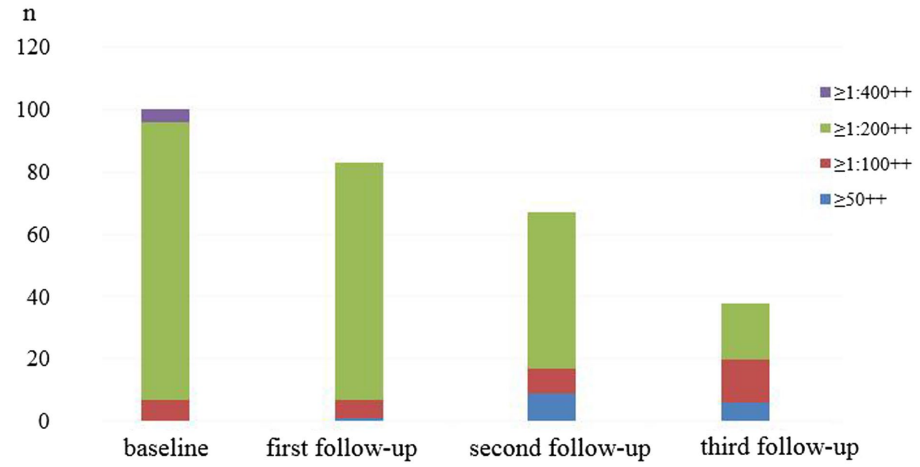

FIGURE 1
Antibody titer composition of SAT test at each survey time.

TABLE 2 Tube agglutination test results at each investigation time point (*n*, %).

	Baseline survey	First follow-up	Second follow-up	Third follow-up	X^2	*p*
≥1:100^{++}	7 (7.00)	6 (7.30)	8 (13.79)	14 (43.75)	11.853	0.042
≥1:200^{++}	89 (89.00)	76 (92.70)	50 (86.21)	18 (56.25)	121.251	<0.001
≥1:400^{++}	4 (4.00)	0 (0)	0 (0)	0 (0)	12.121	0.007
Total	100 (100)	82 (100)	58 (100)	32 (100)		

Data in the table represent the number of cases (constituent ratio %); chi-square test was used to compare the results of tube agglutination test at each time point.

TABLE 3 Tube agglutination test results in different disease course (*n*, %).

	Acute phase (*n* = 100)	Subacute phase (*n* = 21)	Chronic phase (*n* = 11)	X^2	*p*
≥1:100^{++}	7 (7.00)	9 (42.86)	5 (45.45)	24.510	<0.001
≥1:200^{++}	89 (89.00)	12 (57.14)	6 (54.55)	12.235	0.002
≥1:400^{++}	4 (4.00)	0 (0)	0 (0)	2.302	0.316
Total	100 (100)	21 (100)	11 (100)		

Data in the table represent the number of cases (constituent ratio); chi-square test was used to compare the results of tube agglutination test at each time point.

proportion, the proportion of patients with an antibody titre ≥1:100^{++} in the chronic phase was higher than that in the subacute phase and acute phase, and the proportion of patients with an antibody titre ≥1:200^{++} in the acute phase was higher than that in subacute phase and chronic phase. See Table 3 for details.

4.3. Liver function test results

All included patients underwent unified and standardised treatment, and liver function, renal function, and haematologic system tests were performed at each survey time point. Compared with the baseline survey, the proportion of patients with abnormal liver function test results increased at the first (89 patients, 89%) and second follow-up (94 patients, 94%) visits, and the proportion of patients with abnormal liver function test results at the third follow-up (27 patients, 27%) visit was much lower than that at the first two follow-up visits ($p < 0.01$); the trends of AST and TBIL were generally consistent ($p < 0.01$).

4.4. Renal function test results

Compared with the baseline investigation, the proportion of patients with abnormal renal function decreased to 13% at the third follow-up visit. Among them, the proportion of patients with elevated BUN increased at the first visit (22 patients, 22%) and the second visit (15 patients, 15%) ($p < 0.01$); those with elevated SCr began to decrease at the second visit (11 patients, 11%), falling to only 2 patients with elevated SCr at the third visit ($p < 0.01$).

4.5. Haematologic test results

Compared with the baseline survey, the proportion of those with haematologic abnormalities decreased at the first (60 patients, 60%) and second follow-up (49 patients, 49%) ($p < 0.05$) visits, but this number increased again at the third follow-up (69 patients, 69%) visit. The proportion of patients with abnormal RBC count

TABLE 4 Laboratory test results of brucellosis patients at each investigation time point.

Exam	Specific indicators	Baseline survey (case)	First follow-up (case)	Second follow-up (case)	Third follow-up (case)	X^2	p
Liver function	ALT (0–40 U/L) increased	13	14	21	5	11.201	0.011
	AST (0–40 U/L) increased	11	27	23	11	13.821	0.003
	TBIL (3.4–20.5 umol/L) increased	24	48	50	11	48.605	<0.001
	Total	48	89	94	27	137.526	<0.001
Renal function	BUN (2.9–8.2 mmol/L) increased	4	22	15	11	12.866	0.005
	SCr (35–115 umol/L) Increased	29	22	11	2	31.696	<0.001
	Total	33	44	26	13	24.575	<0.001
Hematologic	RBC (3.5–5.5 × 1,012/L) increased	10	3	5	9	15.203	0.058
	WBC (4–10 × 10 ^{9}/L) decreased	22	21	20	21	0.121	0.989
	Hb (110–160 g/L) increased	34	31	24	34	23.135	0.0371
	PLT (100–300 × 10 ^{9}/L) decreased	15	5	0	5	20.267	<0.001
	Total	81	60	49	69	24.218	<0.001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, bilirubin; BUN, blood urea nitrogen; SCr, serum creatinine; RBC, red blood cell; WBC, white blood cell; Hb, hemoglobin; PLT, platelet; chi-square test was used to compare the laboratory test results of patients at each time point.

TABLE 5 Mauchly's test of sphericity.

Within-subjects effect (time)	Mauchly's W	Approximate chi-square	Degrees of freedom	p	Eplison		
					Greenhouse–Geisser	Hunyh–Feldt	Lower bound
ALT	0.137	32.250	5	0.051	0.390	0.392	0.333
AST	0.891	11.264	5	0.046	0.938	0.968	0.333
TBIL	0.010	443.390	5	0.037	0.361	0.362	0.334
BUN	0.113	567.038	5	0.044	0.402	0.404	0.333
SCr	0.290	120.811	5	0.043	0.599	0.610	0.333
RBC	0.013	540.521	5	0.005	0.369	0.370	0.332
WBC	0.003	538.018	5	0.001	0.368	0.370	0.333
Hb	0.665	39.866	5	0.047	0.808	0.830	0.335
PLT	0.850	15.924	5	0.007	0.918	0.947	0.433

ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, bilirubin; BUN, blood urea nitrogen; SCr, serum creatinine; RBC, red blood cell; WBC, white blood cell; PLT, platelet.

decreased to 3% ($n = 3$) at the first follow-up visit and increased again ($p < 0.05$) at the third follow-up ($n = 9$, 9%) visit; the proportion of patients with elevated Hb decreased at the first ($n = 31$, 31%) and second follow-up ($n = 24$, 24%) visits and increased ($p < 0.05$) at the third follow-up ($n = 34$, 34%) visit. The proportion of patients with decreased PLT count began to decrease at the first follow-up visit ($n = 5$, 5%) ($p < 0.05$). The laboratory test results of patients with brucellosis at each survey time point are shown in Table 4.

The biochemical parameter data at each survey time point were normal with equal variance, and the results of the sphericity test are shown in Table 5. Repeated measures ANOVA showed statistically significant differences in ALT, AST, TBIL, SCr and PLT levels at different time points ($p < 0.05$), as is shown in Table 6. Pairwise comparisons of biochemical indicator levels at the four time points showed that ALT levels were higher at both the first and second follow-up visits than at the third follow-up ($p < 0.01$) visit; in addition, AST levels were higher at the first and second follow-up visits than at the baseline survey and the third follow-up ($p < 0.01$) visit. The TBIL levels were increased at the first and second follow-up visits ($p < 0.01$) compared with the baseline survey, and the SCr levels decreased at the second follow-up visit compared with the first follow-up visit. Additionally, the SCr levels at the third follow-up visit were lower than that at the baseline survey and the first and second follow-up ($p < 0.01$) visits. The PLT levels were increased at the first and the second follow-up visit compared with the baseline survey but started to decrease at the third follow-up ($p < 0.01$) visit compared with the second follow-up visit. These results are shown in Table 7.

The ANOVA laboratory biochemical indicators showed that the ALT levels in the acute phase of brucellosis were lower than those in the subacute and chronic phases ($p < 0.01$). In addition, there were no significant differences in the AST, TBIL, BUN, SCr, RBC, WBC, Hb and PLT levels in the acute, subacute and chronic phases of brucellosis ($p > 0.05$). See Table 8.

TABLE 6 Repeated biochemical measurements at each survey time point.

	Sum of squares of deviation from mean	Degrees of freedom	Mean square	*F*	*p*
ALT	8299.14	3	2766.38	2.014	0.042
AST	2219.42	2.813	789.12	3.370	0.022
TBIL	4035.34	1.084	1345.114	0.442	0.047
BUN	8536.99	1.206	2845.67	0.973	0.406
SCr	1101.497	1.798	3670.832	14.635	<0.001
RBC	232.04	1.107	209.561	1.010	0.325
WBC	220.457	1.105	199.45	10.30	0.321
Hb	329.648	2.425	135.916	0.186	0.869
PLT	455.437	2.755	165.981	7.973	<0.001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, bilirubin; BUN, blood urea nitrogen; SCr, serum creatinine; RBC, red blood cell; WBC, white blood cell; PLT, platelet.

TABLE 7 Results of pairwise comparison of biochemical indicators at each investigation time point.

		Baseline survey ($\bar{X} \pm S$, $n = 100$)	First follow-up ($\bar{X} \pm S$, $n = 100$)	Second follow-up ($\bar{X} \pm S$, $n = 100$)	Third follow-up ($\bar{X} \pm S$, $n = 100$)	*F*	*p*
Liver function	ALT	32.567 ± 64.430	28.777 ± 17.144	29.092 ± 20.154	20.073 ± 14.354	12.519	0.001
	AST	26.994 ± 16.277	32.159 ± 18.230	30.966 ± 17.453	26.807 ± 16.203	3.688	0.005
	TBIL	16.308 ± 11.630	22.815 ± 16.153	22.415 ± 14.920	24.977 ± 107.349	4.67	0.006
Renal function	BUN	8.173 ± 34.880	17.432 ± 102.147	6.547 ± 4.532	6.0425 ± 2.094	0.859	0.465
	SCr	83.906 ± 83.344	96.094 ± 45.914	77.167 ± 29.717	51.073 ± 26.633	25.145	0.001
Hematologic system	RBC	6.233 ± 16.421	4.817 ± 3.926	4.440 ± 0.679	4.378 ± 0.827	0.848	0.471
	WBC	5.061 ± 1.640	5.249 ± 1.539	5.232 ± 1.575	5.296 ± 3.014	0.421	0.738
	Hb	152.700 ± 33.081	150.96 ± 21.162	150.21 ± 23.415	151.42 ± 29.302	0.160	0.923
	PLT	154.885 ± 59.434	157.83 ± 50.074	184.16 ± 47.601	171.82 ± 49.867	7.513	0.001

ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, bilirubin; BUN, blood urea nitrogen; SCr, serum creatinine; RBC, red blood cell; WBC, white blood cell; PLT, platelet.

TABLE 8 Laboratory biochemical parameters in patients with brucellosis in acute, subacute and chronic phases.

	Acute phase ($\bar{X} \pm S$, $n = 100$)	Subacute phase ($\bar{X} \pm S$, $n = 21$)	Chronic phase ($\bar{X} \pm S$, $n = 11$)	*F*	*p*
ALT	25.015 ± 13.094	16.175 ± 6.706	25.173 ± 9.877	4.52	<0.001
AST	25.687 ± 17.269	23.530 ± 7.147	29.491 ± 9.735	0.54	0.579
TBIL	16.526 ± 12.355	15.071 ± 8.871	13.240 ± 4.283	0.481	0.697
BUN	9.315 ± 39.984	6.408 ± 2.497	6.218 ± 1.907	0.084	0.933
SCr	88.201 ± 83.427	56.800 ± 20.172	66.455 ± 16.670	1.747	0.056
RBC	6.751 ± 18.823	4.603 ± 0.653	4.250 ± 0.901	0.228	0.701
WBC	5.000 ± 1.760	5.001 ± 1.297	4.780 ± 1.578	0.086	0.941
Hb	149.092 ± 32.599	157.950 ± 20.353	152.182 ± 30.558	0.675	0.552
PLT	155.125 ± 61.923	183.500 ± 40.841	147.455 ± 46.231	2.238	0.522

ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, bilirubin; BUN, blood urea nitrogen; SCr, serum creatinine; RBC, red blood cell; WBC, white blood cell; Hb, hemoglobin; PLT, platelet.

4.6. Clinical symptoms

At baseline, the main clinical manifestations of 100 patients with brucellosis were joint pain (81 cases, 81.0%), fatigue (55 cases, 55.0%) and fever (40 cases, 40.0%). Patients with joint pain mainly presented with pain in the knee joint (42 cases, 51.9%), shoulder joint (30 cases, 37.0%) and wrist joint (23 cases, 28.4%) and often presented with polyarticular discomfort (49 cases, 60.5%). At the first follow-up visit, joint pain (55 cases, 55.0%), muscle pain (27 cases, 27.0%) and fatigue (27 cases, 27.0%) were the main manifestations, and knee pain (24 cases, 43.6%) was more significant in patients with joint pain. In addition, compared with the baseline survey, those with fever (15 cases, 15.0%) and polyarticular pain (19 cases, 34.6%) were reduced ($p < 0.01$). At the second follow-up visit, joint pain (39 cases, 39.0%),

TABLE 9 Clinical symptoms of brucellosis patients at each investigation time point.

	Baseline survey (e.g., $n = 100$)	First follow-up (e.g., $n = 100$)	Second follow-up (e.g., $n = 100$)	Third follow-up (e.g., $n = 100$)	X^2/Z	p
Fever (>37.2°C)	40	15	9	2	59.590	<0.001
Asthenia	55	27	25	6	60.325	<0.001
Hyperhidrosis	38	9	13	1	59.171	<0.001
Arthralgia	81	55	39	19	29.688	<0.001
Sacrum	15	10	5	3	11.461	0.009
Ilium	7	1	4	3	5.195	0.158
Shoulder	30	8	8	6	34.306	<0.001
Genu	42	24	13	4	48.816	<0.001
Elbow	20	1	2	0	50.329	<0.001
Wrist	23	13	4	1	32.040	<0.001
Ankle	15	12	6	2	12.869	0.005
Spine	1	0	0	0	3.008	0.390
Monoarticular	25	31	24	14	8.288	0.04
Polyarticular	49	19	9	3	78.250	<0.001
Migratory	7	5	6	2	2.947	0.400
Muscular pain	37	27	13	2	43.877	<0.001
Chills	6	4	2	2	3.257	0.354
Cold intolerance	9	6	5	1	6.584	0.086
Cough	12	10	2	0	18.440	0.004
Expectoration	6	2	1	0	9.891	0.02
Sleep disorder	8	0	2	1	14.489	0.002

Chi-square test or Fisher exact test was used to compare frequency of clinical symptoms at each time point.

fatigue (25 cases, 25.0%) and hyperhidrosis (13 cases, 13.0%) were predominant and knee pain (13 cases, 33.3%) was more significant in patients with joint pain; those with fever symptoms (9 cases, 9.0%) and polyarticular pain (9 cases, 9.0%) were reduced compared with those at the baseline survey ($p < 0.01$). Joint pain (19 patients, 19.0%) was the most predominant manifestation at the third follow-up visit and was most prominent in patients with shoulder pain (6 patients, 31.6%); there were fewer patients with fever (2 patients, 2.0%), polyarticular pain (3 patients, 15.8%) and muscle pain (2 patients, 2.0%) ($p < 0.01$) compared with the baseline survey (see Table 9).

Joint pain (81 cases, 81.0%), fatigue (55 cases, 55.0%) and fever (40 cases, 40.0%) were the main clinical symptoms in the acute stage. The clinical symptoms of patients with different courses of the disease were analysed. The proportion of patients with hyperhidrosis, chills, fear of cold, cough and expectoration in the acute phase (38.0%, 2.0%, 7.0%, 12.0% and 6.0%) was higher than that in subacute and chronic phases ($p < 0.01$). Compared with patients with acute fever, the proportion of patients with subacute fever (20 patients, 64.5%) increased, and the proportion of patients with joint pain decreased (2 patients, 9.5%). Patients with fever in the chronic phase (1 case, 9.1%) were lower than those in the acute and subacute phases ($p < 0.01$); the proportions of patients with fatigue (3 cases, 27.3%), joint pain (4 cases, 36.4%) and muscle pain (1 case, 9.1%) in the chronic phase were lower than in the acute phase (55.0%, 81.0% and 37.0%, respectively) and higher than in the subacute phase (9.5%, 9.5% and 0.0%, respectively) ($p < 0.01$). See Table 10.

4.7. Factors affecting the prognosis of patients with brucellosis

4.7.1. Univariate logistic regression analysis

Of the 100 patients, 68 were cured and 32 were uncured at the third follow-up visit. Univariate logistic regression was used to analyse gender, age, clinical symptoms and laboratory test results; the results are shown in Table 11. Gender was not a factor influencing poor prognostic outcome (not cured) [$p = 0.986$, 95% confidence ratio (CI) = 0.311–3.281], and patients aged ≥45 years had a higher risk of a poor prognostic outcome (not cured) than patients younger than 45 years old [$p = 0.021$, odds ratio (OR) = 6.336, 95% CI = 1.318–30.458]. Comparing the laboratory test results between the two groups, patients with elevated SCr ($p = 0.015$, OR = 1.076, 95% CI = 0.010–0.062) and patients with elevated Hb had a higher risk of a poor prognostic outcome (not cured) ($p = 0.009$, OR = 4.918, 95% CI = 1.484–16.295). In addition, there was no statistically significant difference in the proportion of fatigue, shoulder and knee pain, type of joint pain, whether the medication was taken on time, and abnormal ALT, AST, RBC, WBC, and PLT results between the two groups ($p > 0.05$).

4.7.2. Multivariate logistic analysis

Age, elevated SCr and elevated Hb were known to be risk factors for a poor prognosis in patients with brucellosis by univariate logistic analysis, and these three variables and risk factors that may trigger

TABLE 10 Clinical symptoms of brucellosis patients with different course.

	Acute phase (n = 100)	Subacute phase (n = 21)	Chronic phase (n = 11)	X^2/Z	p
Fever (>37.2°C)	40	20	1	17.851	<0.001
Asthenia	55	2	3	23.955	0.001
Hyperhidrosis	38	0	0	17.076	0.002
Arthralgia	81	2	4	44.123	<0.001
Sacrum	15	0	0	5.415	0.067
Ilium	7	0	0	4.978	0.083
Shoulder	30	2	3	4.165	0.125
Genu	42	0	1	16.952	0.002
Elbow	20	0	0	12.206	0.002
Wrist	23	0	0	8.913	0.012
Ankle	15	0	0	33.730	<0.001
Spine	1	0	0	0.322	0.851
Monoarticular	25	2	2	2.525	0.283
Polyarticular	49	0	0	24.937	<0.001
Migratory	7	0	1	1.687	0.43
Muscular pain	37	0	1	13.861	0.001
Chills	2	0	0	12.361	0.002
Cold intolerance	7	0	0	14.533	0.001
Cough	12	0	0	16.810	0.002
Expectoration	6	0	0	14.091	0.001
Sleep disorder	8	0	0	14.980	0.001

Chi-square test or Fisher exact test was used to compare frequency of clinical symptoms at each time point.

chronicity in patients were subsequently analysed by multivariate logistic analysis. The results are shown in Table 12. The results showed that joint pain (p = 0.001, OR = 3.652, 95% CI = 1.379–9.672), single joint pain (p = 0.001, OR = 6.356, 95% CI = 4.660–8.669), elevated SCr (p = 0.017, OR = 15.804, 95% CI = 1.644–151.966) and elevated Hb (p = 0.014, OR = 1.219, 95% CI = 0.065–1.736) were risk factors for uncured patients. The other variables included in the multivariate logistic analysis showed no significant difference between the two groups (p > 0.05).

5. Discussion

Brucellosis is a widespread global zoonosis disease and has been found in humans and animals in more than 170 countries (4, 13); it is primarily concentrated in countries in the Middle East, along the Mediterranean coast, and in sub-Saharan Africa, Asia, and South America (14–16). In many provinces (municipalities directly under the central government and autonomous regions) in China, there are varying degrees of occurrence and prevalence of brucellosis. The most serious issue occurred in the 1950s and 1960s, with a steady decline in the 1970s and 1980s. However, cases showed a rising trend in the middle and late 1990s, resulting in an epidemic situation going into the 21st century. At present, brucellosis in China is mainly distributed in provinces and regions engaged in the animal husbandry and breeding industry in southwest and northwest China, and the incidence has been stable at about 20/100,000 for nearly five consecutive years (17).

Inner Mongolia is a historical epidemic area of brucellosis. In the 1980s, the epidemic situation was effectively controlled. However, since the late 1990s, especially into the 2000s, the epidemic has rebounded rapidly because of the vigorous development of animal husbandry. In 2017, there was a steep rise with 7,744 new cases reported in the region. In 2018, 10,111 cases were reported, and the number continued to rise from there: 14,148 in 2019, 17,478 in 2020, and 21,910 in 2021. In 2021, the number of cases in Inner Mongolia accounted for 51.32% of the total number of cases reported in China, ranking first in the country. This was also a record high for reported cases in the region. In fact, since 2017, the number of reported cases in Inner Mongolia has ranked first in China for five consecutive years, and the annual growth rate of the number of cases from 2017 to 2021 was 29.49%. This has seriously affected the health and economic development of the people in the region and has become a major public health issue (18, 19).

In this study, 100 patients with acute brucellosis were followed up with for 6 months. The differences in laboratory test results over the different disease stages were repeatedly measured, and the changes in serum and biochemical parameters during this time were analysed. This study also explored the risk factors affecting the prognosis of patients with brucellosis.

A total of 100 patients with brucellosis were included, and the animals these patients were exposed to were mainly cattle, sheep, pigs,

TABLE 11 Univariate logistic regression analysis of adverse prognostic outcomes in brucellosis patients.

Independent variable	Cured (n = 68)	Not cured (n = 32)	p	OR	95% CI
Female	31 (47.69)	11 (31.43)	0.986	1.011	0.311–3.281
Age ≥45 years	48 (73.85)	32 (91.43)	0.021	6.336	1.318–30.458
Asthenia	3 (4.62)	3 (8.57)	0.894	1.215	0.070–21.226
Arthralgia	15 (23.08)	4 (11.43)	1.000	3.541	2.156–7.312
Shoulder pain	4 (6.15)	2 (5.71)	0.620	2.213	0.096–51.255
Knee pain	3 (4.62)	1 (2.86)	0.429	4.784	0.99–232.175
Type of joint pain					
Monoarticular pain	12 (18.46)	2 (5.71)	1.000	4.213	1.662–9.583
Polyarticular pain	2 (3.08)	0 (0)	0.999	1.324	0.547–4.783
Joint migratory pain	1 (1.54)	1 (2.86)	1.000	1.631	1.242–7.894
On-time medication	9 (13.85)	1 (2.86)	0.395	2.058	0.390–10.872
ALT increased	5 (7.70)	1 (2.86)	0.831	0.818	0.130–5.150
AST increased	8 (12.31)	3 (8.57)	0.598	0.494	0.036–6.785
TBIL increased	14 (20.59)	4 (12.50)	0.348	0.548	0.156–1.924
BUN increased	10 (14.71)	7 (21.88)	0.586	1.396	0.420–4.635
SCr increased	19 (32.76)	1 (2.86)	0.015	1.076	0.010–0.062
RBC increased	9 (13.85)	5 (14.29)	0.892	1.120	0.218–5.759
WBC decreased	13 (20.00)	10 (28.57)	0.059	3.501	0.955–12.829
Hb increased	18 (27.69)	16 (45.71)	0.009	4.918	1.484–16.295
PLT decreased	3(4.62)	32(91.43)	0.977	0.969	0.111–8.466

Data in the table represent the number of cases (proportion); ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, bilirubin; BUN, blood urea nitrogen; SCr, serum creatinine; RBC, red blood cell; WBC, white blood cell; Hb, hemoglobin; PLT, platelet.

TABLE 12 Multivariate logistic regression analysis of adverse prognostic outcomes in patients with brucellosis.

Variable	p	OR	95% CI
Female	0.986	1.011	0.311–3.281
Age ≥45 years	0.144	0.234	0.036–1.626
Asthenia	0.833	0.806	0.045–14.324
Arthralgia	0.001	3.652	1.379–9.672
Shoulder pain	0.324	0.172	0.005–5.658
Knee pain	0.429	0.193	0.003–11.363
Monoarticular pain	0.001	6.356	4.660–8.669
Polyarticular pain	0.997	9.522	6.730–11.641
Joint migratory pain	0.661	4.856	3.611–6.713
On-time medication	0.434	0.517	0.099–2.706
ALT increased	0.876	1.224	0.097–15.390
AST increased	0.951	1.060	0.167–6.714
TBIL increased	0.149	3.111	0.665–14.554
BUN increased	0.404	0.500	0.098–2.542
SCr increased	0.017	15.804	1.644–151.966
RBC increased	0.967	0.966	0.188–4.958
WBC decreased	0.077	0.305	0.082–1.135
Hb increased	0.014	1.219	0.065–1.736
PLT decreased	0.992	1.011	0.118–8.691

ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, bilirubin; BUN, blood urea nitrogen; SCr, serum creatinine; RBC, red blood cell; WBC, white blood cell; Hb, hemoglobin; PLT, platelet.

and dogs. In addition, 98.98% were exposed to two or more animals at the same time. The patients had an average age of 53.810 ± 0.993 years, and the age of onset and gender characteristics of brucellosis were consistent with previous studies (20, 21). In this study, the average age of disease onset was 53.8 years, which may be related to the fact that rural residents over 50 years of age choose to work in agriculture at home and raise livestock during farming leisure time. In addition, elderly adults have less physical strength than younger adults, and brucellosis awareness was lower in the elderly than in the young population (22). We similarly observed gender differences in this study, including a higher proportion of male patients than females.

In the SAT test, 100 patients in the acute phase were treated by standardised unified therapy. At the first follow-up, 4 patients had SAT agglutination antibody titers ≥1:400^{++}, and 89 patients had SAT agglutination antibody titers ≥1:200^{++}; at the third follow-up, patients with no antibody titers ≥1:400^{++}, and those with antibody titers ≥1:200^{++} decreased significantly. García Casallas et al. (23) stated that brucellosis can develop and persist as a chronic disease, becoming a granulomatous disease capable of affecting any organ system. It is evident that the early diagnosis and standardised treatment of patients with brucellosis in the acute phase is essential.

Brucellosis is a systemic infectious disease, which often involves a variety of organs and systems, causing multiple organ-destructive diseases in patients, often resulting in complications. Both ALT and AST are involved in various physiological and biochemical metabolic reactions in the body; the anaerobic glycolysis of glucose leads to the increase of transaminases, so they are commonly used to reflect liver function, and their ratio can predict a variety of tumours and their prognoses (24, 25). Compared with the baseline investigation, there were significantly more patients with abnormal liver function levels and increased AST and TBIL levels during the first and second follow-up visits. This may be related to the fact that the patients with brucellosis were in the acute progression of brucellosis at this time (26–28). With treatment, the proportion of patients with abnormal liver function test results gradually decreased, decreasing to 27% at the third follow-up visit. Compared with the results at the first and second follow-up visits, the ALT and AST levels decreased. These results are consistent with the results of Hosseini SM et al. (29), reporting an increase of 26% in AIT enzyme and 25% in AST enzyme in rats after infection. Another study (30) also reported increased levels of liver enzymes in rats with chronic brucellosis and stage. The Kazak et al. (31) study had similar findings: patients presented with abnormally high AST and ALT levels, noting that the rate of liver involvement in brucellosis was high and that high levels of AST and ALT cannot be ignored. The positive rate of SAT and the antibody titre in 100 patients with acute brucellosis decreased gradually with the course of the disease, and the ALT level in patients with subacute brucellosis was lower than that in patients with acute and chronic brucellosis. There was a certain trajectory in the changes between the two, which provided a theoretical basis for finding markers associated with chronic brucellosis.

Brucellosis is preventable and treatable. In the 100 patients with acute brucellosis included in this study, the main clinical manifestations were joint pain (81%), fatigue (55%), and fever (40%) at the baseline survey, which was consistent with what was noted in a systematic evaluation study by Zheng et al. (32) After receiving unified standardised treatment, the relevant symptoms were gradually reduced or eliminated, and patients whose symptoms manifested as fever, fatigue, hyperhidrosis, and polyarticular pain had been reduced at the first follow-up visit. The proportion of patients with joint pain decreased at the third follow-up visit, and the number of patients complaining of shoulder joint and elbow joint pain decreased at the first follow-up visit. Therefore, the relevant clinical symptoms of the patients in the acute phase of brucellosis can be effectively relieved and eliminated through a full course of standardised treatment.

The results of this study showed that there were significant differences in the AST, ALT and PLT results at the baseline survey and subsequent follow-up visits.

Under the influence of *Brucella*, chronic body consumption and hypersplenism often accompany an abnormal haemogram (33, 34). Creatinine is a measure of glomerular filtration function and the risk of chronicity increases when SCr increases. An abnormal haemogram is generally a transient complication, which is easily converted after standardised treatment. Haemoglobin is a protein that transports oxygen in RBCs, and this study's results show that when Hb is elevated in patients with brucellosis and not treated timely, the risk it of chronicity increases. In the present study, there were no significant differences in the RBC, WBC, Hb and PLT levels in the acute, subacute, and chronic phases of brucellosis ($p > 0.05$), which is in agreement with the literature (35). However, in Hosseini's et al. (29) study, the number of WBCs was increased by 21% in rats during the acute phase of the infection. The difference between results could be due to the different study subjects. The chronicity rate of brucellosis in this study was 11%, and the logistic regression analysis of the risk factors affecting the prognosis of brucellosis revealed that an age ≥45 years, joint pain, and single joint pain were risk factors for patients who were not cured. In addition, some patients had elevated SCr and Hb levels, and if left untreated, elevated SCr and Hb were also risk factors for patients who were not cured. The results of the multivariate logistic regression analysis showed that joint pain, single joint pain and elevated SCr and Hb were all risk factors for adverse outcomes. When other factors remained unchanged, the probability of chronicity increased by a factor of 3.652 ($p < 0.05$) in patients with arthralgia symptoms and by a factor of 6.356 ($p < 0.05$) in patients presenting with single joint pain. If patients with elevated SCr and Hb were not monitored and treated timely, the risk of chronicity increased by a factor of 15.804 ($p < 0.05$) in patients with elevated SCr and by a factor of 1.219 ($p < 0.05$) in patients with elevated Hb. Therefore, according to the risk factors, it is recommended that the treatment cycle should be extended for such patients.

This study had some limitations. First, this study only investigated the risk factors affecting the prognosis of brucellosis; it cannot be concluded that there is any causal association. Second, the sample source of this study is relatively single-centre, and the conclusions drawn may only be regional. The next step is to expand the size of the study and establish a cohort for a long-term, in-depth study.

6. Conclusion

The trajectory of changes in patient SAT posirates and antibody titers can be used to distinguish patients with chronic brucellosis. Brucellosis is preventable and treatable, and a standardised treatment can be effective in reducing the clinical symptoms of patients. If patients are not treated in a timely manner, joint pain, monoarticular pain, and elevated SCr are risk factors for patients who are not cured. Therefore, the treatment cycle for these patients should be extended.

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 studies involving humans were approved by the Ethics Committee of the Center for Disease Control and 463 Prevention of Inner Mongolia Autonomous Region (Approval No.: 2022011601). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YW and HB designed the study. LB, NT, AZ, HM, XL, and BW performed clinical examinations and collected patient data. LB and NT drafted the manuscript. All authors read and approved the submitted version of the 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.

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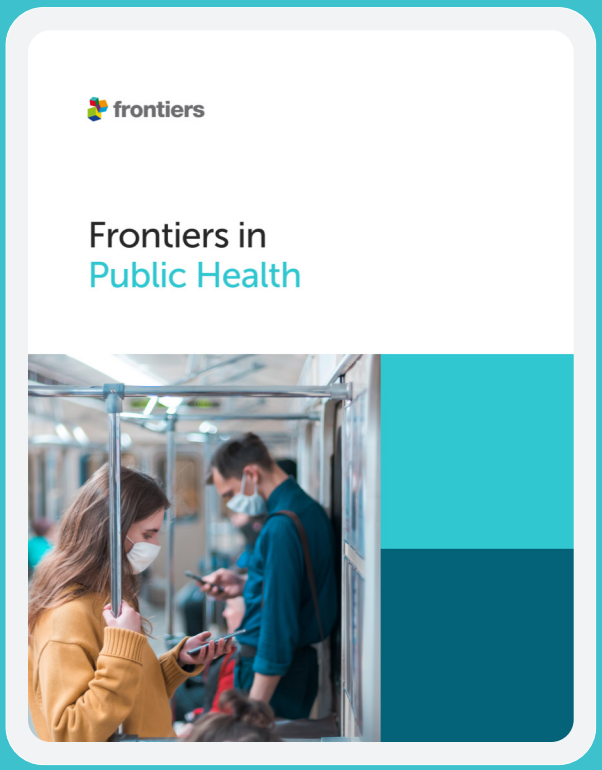

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