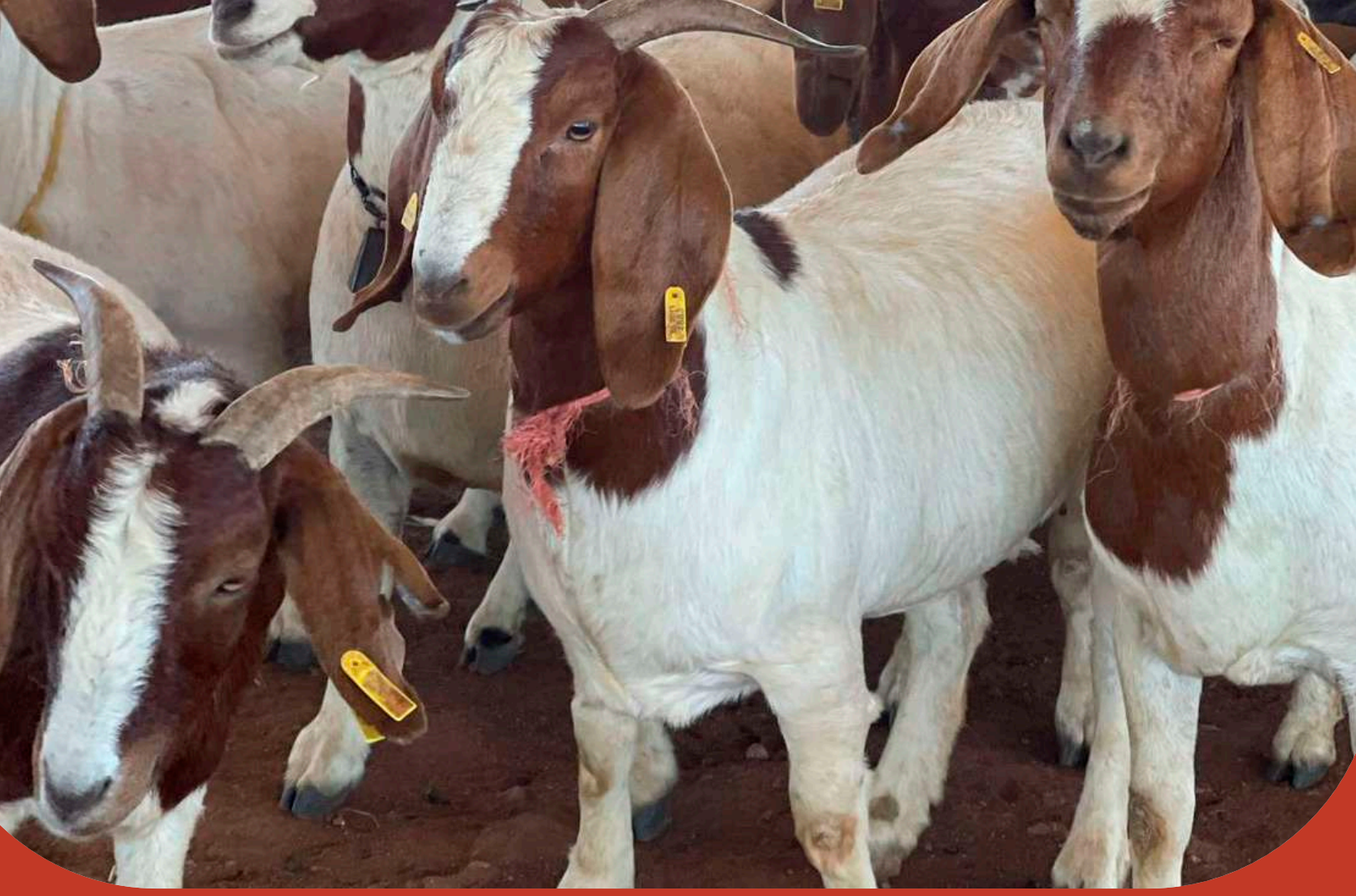

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ORIGINAL ARTICLE

Retrospective seroepidemiological study of small ruminant lentivirus, paratuberculosis and brucellosis in goats from Mexico, based on multiplex assay

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ABSTRACT. Mexican goat production systems face infection risks from *Brucella melitensis*, small ruminant lentivirus (SRLv) and *Mycobacterium avium* subsp. *paratuberculosis* (MAP); agents that cause great economic losses and directly affect public health (brucellosis and paratuberculosis [PTb]). Currently, there are no diagnostic tests applicable at large scale nor epidemiological information regarding the seroprevalence for these infectious diseases. For this study, a multiplex antibody assay (Luminex®) was used to retrospectively study the seroprevalence of brucellosis, SRLv, and PTb in the sera of 983 goats from nine Mexican states. Sera were obtained between 2014 and 2019. Antibodies against all three infectious diseases were detected in almost all tested samples. The estimated seroprevalence values ranged from 37% to 78% for brucellosis, 21% to 65% for SRLv, and 0% to 13% for PTb. The multiplex assay (Luminex®) is a simple, accessible, efficient, and cost-effective seroprevalence monitoring tool for brucellosis, SRLv, and PTb, and can be used as a large-scale approach.

Keywords: Caprine, diagnostic, antibodies, Luminex®, serological.

INTRODUCTION

Goat production in Mexico is traditionally carried out in economically poor regions, particularly arid and semi-arid regions. This activity represents one of the main sources of income for the population in these regions (Secretaría de Agricultura y Desarrollo Rural, 2016). The majority of goat herds are destined for self-sustenance and are characterized by low levels of technology implementation. For example, there is a lack of health records, grouping according to age or production stage, and it is common practice to keep them in shared communal pasturelands (Cuellar *et al*., 2012). The latter favors contact between animals from different herds and other animal species (e.g., cattle and sheep), which increases the risk of infection and compromises the control of relevant infectious diseases such as brucellosis, small ruminant lentivirus (SRLv), and paratuberculosis (PTb).

Since 2010, the National Council for Animal Health (CONASA, Consejo Técnico Consultivo Nacional de Sanidad Animal) has designated brucellosis and PTb, which are potentially zoonotic diseases, and small ruminant lentivirus and mycoplasmosis, as relevant diseases (CONASA, 2011).

Brucella melitensis is the main cause of brucellosis in goats (Garin -Bastuji *et al*., 1998; Aguilar *et al*., 2011; Díaz-Aparicio, 2013), characterized by abortions towards the end of gestation and occasionally orchitis or epididymitis (Chand *et al*., 2002). This agent, *B. melitensis*, is considered to be of public health importance as it is the most pathogenic *Brucella* spp. in humans (Méndez *et al*., 2015). In Mexico, two diagnostic tests are commonly used and recommended for the determination of caprine brucellosis: agglutination tests using *B. abortus* strain 1119-3, and complement fixation as a confirmatory test (Norma Oficial Mexicana, 1995). Furthermore, a native hapten (NH) from *B. melitensis* has been developed to differentiate vaccinated from naturally infected animals, with the aim of targeting vaccination and other control strategies in different herds (Moreno, *et al*., 1987; Zygmunt *et al*., 1988; Díaz-Aparicio *et al*., 1996).

Small ruminant lentivirus infects sheep and goats worldwide, causing multisystemic chronic and progressive disease, characterized by pneumonia, encephalitis, arthritis, and mastitis (Minguijón *et al*., 2015). In Mexico, SRLv was officially recognized in goats in the 1990s and sheep in 2016

(Mendiola *et al*., 2019). The main SRLv diagnostic techniques are Agar Gel Immunodiffusion (AGID) and ELISA (Fry *et al*., 2008; Martínez *et al*., 2012; OIE, 2017a), with PCR considered a complementary technique (De Andrés *et al*., 2005; OIE, 2017a).

Paratuberculosis, also known as Johne's disease, is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Robbe, 2011; Fiorentino *et al*., 2012). The clinical disease is characterized by chronic enteritis, decreased milk production, and progressive weight loss, which causes emaciation and death in adult animals (Estévez-Denaives *et al*., 2007; Kheirandish, *et al*., 2009). The most reliable method for the diagnosis of MAP is fecal culture (OIE, 2017b, 2021), but it is hardly used due to the prolonged incubation periods required (> 6 weeks) (Estévez-Denaives *et al*., 2007). ELISA has been proposed as an alternative diagnostic test for seropositive animals (Stabel *et al*., 2009).

Based on the importance of the above-mentioned diseases for Mexican goat production systems, the aim of this study was to conduct a retrospective seroepidemiological study to estimate the prevalence of brucellosis, SRLv, and PTb in goats from different regions of Mexico using a multiplex assay (Luminex®). This test is highly efficient because it reduces the detection time and required sample volumes. Additionally, it's a reliable, reproducible, sensitive, and specific test with high accuracy and precision indices (Ray *et al*., 2005; Elshal & McCoy, 2006; Anderson, 2011; Nájera-Rivera *et al*., 2023) and has been used in the diagnosis of other infectious diseases (Ravindran *et al*., 2010; Anderson *et al*., 2011; Rodriguez *et al*., 2023).

MATERIALS AND METHODS

A total of 983 goat sera obtained from the goat sera bank of the Faculty of Veterinary Medicine (UNAM) were analyzed. The samples correspond to convenience sampling conducted in nine different states of the Mexican Republic between 2014 and 2019. The samples were taken in a few municipalities per state at different times due to the availability of animals and not across the indicated years. Table 1 shows the number of samples analyzed by state and municipality.

To perform the triplex assay (Luminex® Corporation, Texas, USA), the protoplasmic antigen (PPA-3) (Allied Monitor Laboratory, Missouri, US) from MAP, native hapten (NH) from *B. melitensis*, and recombinant proteins p16 and gp38 from SRLv were used. PPA-3 corresponds to a bacterial lysate from the strain *M. avium*, which has been demonstrated

Table 1.
A total of 983 samples obtained from 2014 to 2019 through convenience sampling were analyzed and categorized by state and municipality.

State	Municipality	Number of samples per municipality	Total samples/State
Estado de México	Capultengo	32	48
	Ixtapaluca	16	
Baja California Sur	Comondú	37	37
Coahuila	Matamoros	43	76
	Viesca	33	
Guanajuato	Pénjamo	24	180
	Salamanca	43	
	Santa Cruz de Juventino Rosas	113	
Querétaro	Tequisquiapan	339	339
Sinaloa	Culiacán	81	81
Sonora	Cajeme	22	22
Tlaxcala	Altzayanca	102	102
Veracruz	Altotonga	67	98
	Coatepec	31	
TOTAL		983	983

to be immunogenic, with sensitivity values of up to 80% and specificity of 90–95% (Costanzo *et al.*, 2012). For SRLv, the genes encoding the matrix protein (P16) and transmembrane protein (gp38) from the SRLv genome subtype B were amplified and cloned into an expression vector (Hötzel & Cheevers, 2001; Vázquez Franco, 2011). Native hapten antigen, a 14.5 kDa polysaccharide, was obtained using a previously described method (Moreno *et al.*, 1987; Zygmunt *et al.*, 1988).

The four antigens used comprised a triplex diagnostic panel. The procedure was standardized and previously validated; its sensitivity was between 84.4% and 98.9%, and its specificity was between 95.9% and 98.4% (Nájera-Rivera *et al.*, 2023). In the case of SRLv, sera that reacted with both p16 and gp38 antigens were considered seropositive.

Statistical analysis

Seroprevalence was calculated through the detection of antibodies that recognize each of the antigens used, with a confidence interval of 95%. An imperfect test was performed using EpiR software in the open source R software (R Core Team, 2013) (Rosati *et al.*, 2004; Mark *et al.*, 2023). This statistical test uses the apparent prevalence, as well as the sensitivity and specificity of the diagnostic test to obtain an estimated prevalence value (Rogan & Gladen, 1978), whereas the confidence intervals of the estimated prevalence were obtained using the methodology proposed by Reiczigel *et al.* (2010).

RESULTS

Based on the serological multiplex assay, all the samples were positive for at least one of the diseases tested in this study. Antibodies against the agents studied were found in all sampled Mexican states. In general, brucellosis and SRLv showed a high estimated prevalence (37% and 21%, respectively). Meanwhile, the PTb-estimated seroprevalence was low in almost all states, with Guanajuato having the highest seroprevalence of 13%. The percentages of the estimated seroprevalence and confidence intervals for each state are shown in Table 2. Figure 1 shows the geolocation of the sampled municipalities.

DISCUSSION

This retrospective seroepidemiologic study was conducted in nine Mexican states using the multiplex Luminex® technique to estimate seroprevalences (from 2014 to 2019)

Table 2.
The estimated seroprevalence percentages and confidence intervals were calculated for brucellosis, SRLv, and PTb, by state. The highest estimated seroprevalence was observed in brucellosis and SRLv. Antibodies against these antigens were present in all Mexican states where the samples were collected.

State	Estimated seroprevalence by disease (intervals)		
	Brucellosis	SRLv	PTb
Estado de México	73% (58.53-84.56)	64% (48.83-76.79)	8% (1.06-21.63)
Baja California Sur	71% (53.43-83.58)	21% (9.70-37.01)	5% (-0.96-20.62)
Coahuila	76% (64.09-84.50)	44% (32.42-55.44)	0% (-2.65-8.58)
Guanajuato	67% (59.05-73.60)	54% (46.38-61.65)	13% (8.04-20.20)
Querétaro	55% (49.39-60.60)	57% (51.65-62.75)	2% (-0.54-4.97)
Sinaloa	50% (38.23-60.90)	41% (30.13-52.28)	4% (-0.21-13.54)
Sonora	78% (55.93-91.43)	65% (43.10-82.59)	6% (-1.27-28.21)
Tlaxcala	37% (27.94-47.78)	27% (18.53-36.60)	0% (-2.43-7.03)
Veracruz	74% (63.81-82.19)	44% (34.12-54.53)	2% (-1.66-9.06)
Total	60% (57.08-63.58)	49% (45.59-52.20)	4% (2.60-6.42)

SRLv, Small ruminant lentivirus; PTb, Paratuberculosis.

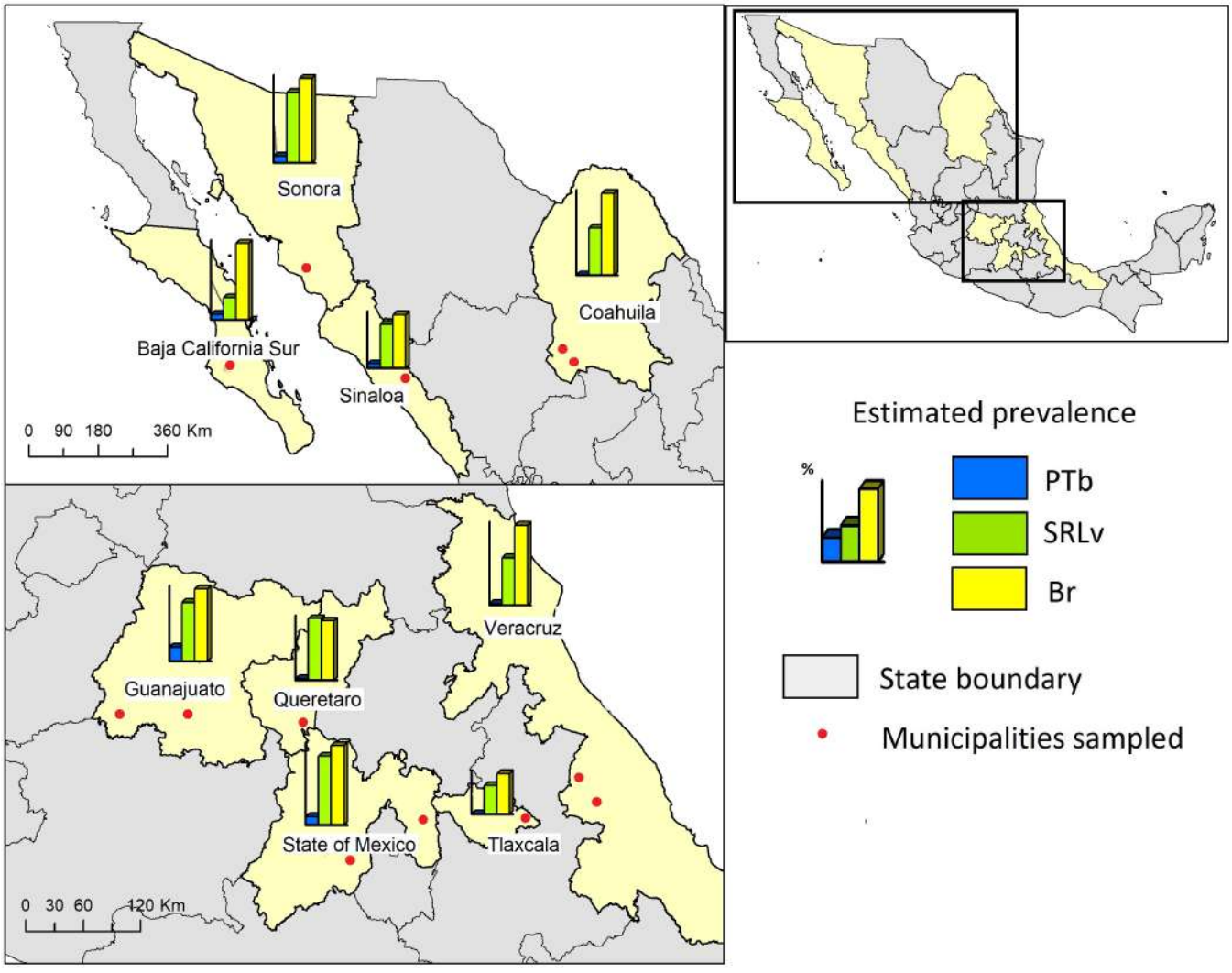

Figure 1.
Map with geolocation of sampled states and municipalities. The estimated seroprevalence and geolocation for brucellosis, SRLv, and PTb are shown in histograms by state. Brucellosis and SRLv had the highest estimated seroprevalence among all sampled states.

for brucellosis, small ruminant lentivirus, and paratuberculosis, which are considered the most relevant infectious diseases in the goat industry in Mexico (Palomares *et al.*, 2021).

The results of this study showed that the three identified diseases had a recurring presence throughout the country. Among the analyzed states, brucellosis (60%) and SRLv (49%) showed the highest total seroprevalence. Despite the detection of antibodies against MAP in most states, the total estimated seroprevalence was low (4%).

The seroprevalence of brucellosis was higher than the national seroprevalence of 0.05% reported by the National Health Service (SENASICA) from the Secretariat of Agriculture (SADER) in 2018 (Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2022). However, this official prevalence report was generated using a non-randomized sampling approach, mostly based on voluntary farmer participation; therefore, a possible sampling bias may underestimate the prevalence of brucellosis in the country. Thus, this sampling approach cannot be used as a valid reference for caprine brucellosis in Mexico.

In our study, brucellosis was present in every state where sampling was carried out and had the highest number of seropositive animals among the three evaluated diseases. Owing to the higher sensitivity of the multiplex assay, it is possible that we identified a higher number of seropositive animals than other tests used to monitor this disease (Elshal & McCoy, 2006; Anderson, 2011).

Of the three diseases evaluated in this study, brucellosis was the only one that had a proper and official routine diagnosis, for which vaccination is mandatory (Norma Oficial Mexicana, 1995). Nevertheless, an important problem when vaccinating goats against brucellosis is that no information

is regularly recorded, that is, animal ID, dose used, or date of immunization. The high prevalence of seropositive samples in this study is due to natural infections caused by field strains and not due to vaccination, since the NH antigen does not detect vaccine antibodies (Díaz-Aparicio *et al*., 1994; Díaz-Aparicio *et al*., 1996).

In Mexico, the predominant SRLv is genotype B1 (Ramírez *et al*., 2011), and the proteins encoded by the lentivirus *gag* gene (capsid and matrix) are used in serological tests that are highly sensitive for the detection of antibodies for prolonged periods of time and early stages of the infection (Grego *et al*., 2005). Additionally, the combined use of these recombinant proteins in ELISA-type tests generates a diagnostic efficiency greater than that obtained individually (Rosati *et al*., 2004). In the present study, the estimated seroprevalence of SRLv was 49% in all the studied Mexican states, comparable to the 53.9% reported in 2021 in six states using ELISA (De la Luz *et al*., 2021). Moreover, the prevalence found in four of the studied states was greater than 50% (Sonora 64%, Estado de México 63%, Querétaro 56%, and Guanajuato 53%), which is similar to previous reports for this state (De la Luz *et al*., 2021). Even in states where we found a lower prevalence, these were still considered high (Tlaxcala 27%, and Baja California Sur 21%). This situation can be explained by the fact that SRLv is not part of any program to control this disease in Mexico. Moreover, the high seroprevalence estimated for SRLv in this study could be due to the high sensitivity of the Luminex ® technique, in which the detection of seropositive animals was determined using two recombinant SRLv proteins of genotype B1, giving the technique a higher probability of detecting true positives. It is also possible that the infection is increasing in the country's herds, since in the 80s, it was estimated that 27% of the animals were seropositive (Nazara *et al*., 1985).

Caprine PTb is an endemic disease widely disseminated in Latin America. However, in Mexico, few studies have determined its seroprevalence in certain regions or states (Mejía *et al*., 2015; Espeschit *et al*., 2017). The highest PTb seroprevalence in this study was 13% in Guanajuato. A study conducted by Favila *et al*. (2009) also found high seroprevalence in the same state (22%). This could be related to the intensive milk production system used, which favors the spread of infection among animals. Conversely, a low PTb prevalence was reported for Guanajuato using a different test, AGID (Meza *et al*., 2019), which is less sensitive than Luminex® used in this study.

The states with the lowest PTb prevalence in our study (< 2%) were Coahuila, Tlaxcala, Veracruz, and Querétaro, comparable to those previously reported for Veracruz (0.6%) (Villagómez *et al*., 2012), but lower than those reported for Coahuila (15%) (Toledo *et al*., 2010). In this study, we observed a total seroprevalence of 4%, with no state showing seroprevalence above 8%, except for Guanajuato. This finding is consistent with that reported by Morales *et al*. (2020), who reported a prevalence of 7% in Sonora. However, in other studies in which ELISA was used, the seroprevalence was higher (Fávila *et al*., 2009, 2010; Toledo *et al*., 2010; Gallaga *et al*., 2017).

These differences may be due to the use of techniques with different sensitivities and specificities, or to a low seroconversion of the animals because the long incubation periods of this infectious agent make its early detection difficult in truly infected animals (Ramírez *et al*., 2011). Therefore, a comparison of the results with previous reports should be performed with caution. Nevertheless, our data indicate that this disease is widespread in Mexico. Adequate control of PTb in the clinical and subclinical stages in animals (Espeschit *et al*., 2017) is of public health importance because it has been associated with Crohn's disease in humans (Sutton *et al*., 2000; Sechi *et al*., 2005; Hermon-Taylor, 2009).

In the present study, it was shown that the use of a multiple diagnostic test, such as Luminex®, with high sensitivity and specificity parameters is functional for large-scale diagnosis. Furthermore, the test is easy, affordable, and allows efficient sample usage.

The sample size used in this study was minimal because they came from a serum bank, obtained by convenience; therefore, the randomization condition that is essential for obtaining reliable epidemiological values was not fully met (Pérez *et al*., 2017). Likewise, the comparison of the data from this study with other reports has the limitation of not knowing the sensitivity and specificity values of each test. However, the estimated prevalence implies that brucellosis, small ruminant lentivirus, and paratuberculosis are present in Mexico. Therefore, it is important to carry out serological monitoring to confirm the current status of these diseases in different regions and herds of the country and thus implement the most effective epidemiological measures to preserve animal and human health.

It is worth noting that the samples tested in our study were taken between 2014-2019, and during this period, several control measures and programs may have been put in place to control the diseases reported here. For example, we described an estimated prevalence of brucellosis in Sonora of 78% in samples collected in 2015, when an eradication program to control the disease was in place. Currently, brucellosis is considered to be eradicated in the State of Sonora (Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria, 2022). Therefore, the present report represents an excellent reference for evaluating the success and effectiveness of control and eradication programs implemented during or after the study period.

CONCLUSION

This study showed that brucellosis, small ruminant lentivirus, and paratuberculosis are common in goat production systems in Mexico. Furthermore, we demonstrated that Luminex® technology is suitable for detecting antibodies against viral and bacterial antigens of high or low prevalence diseases in goat herds.

It is important to continue to monitor the status of these diseases to establish the most viable prevention, control, and eradication measures, according to the region and production system, particularly in the case of brucellosis and paratuberculosis, owing to their implications for public health.

DECLARATIONS

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Author contributions

ADRC: data curation, formal analysis, investigation, methodology, supervision, validation, writing of the original draft, writing review, and editing. CMC: data curation, formal analysis, and investigation. HDNR: data curation, formal analysis, investigation, validation, writing of the original draft, writing review, and editing. EDA: conceptualization, resources, supervision, writing review, and editing. ADP: conceptualization, resources, supervision, writing review, and editing. HRA: conceptualization, resources, supervision, writing review, and editing. ORC: formal analysis and visualization. AZG: formal analysis and visualization. EHL: resources, supervision, writing review, and editing. LCM: conceptualization, funding acquisition, project administration, resources, supervision, writing of the original draft, writing review, and editing.

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ORIGINAL ARTICLE

Physicochemical characteristics and protein profile of oviductal and uterine fluids from domestic sheep

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ABSTRACT. Oviductal (OF) and uterine (UF) fluids are a complex mixture of ions and macromolecules dissolved in water, derived from the secretions of secretory cells and transudates of the circulatory system. Through proteomics, OF and UF have been analyzed in different domestic species throughout the estrous cycle or during the first days of pregnancy. Therefore, the aim of this study was to evaluate the volume, osmolarity, concentration and distribution pattern of proteins, as well as the identification of OVGP1, HSP70 and ezrin proteins for their importance in reproductive physiology, in OF and UF from adult criollo type domestic sheep during the early luteal phase of the estrous cycle. An average of 3.2±1.5 µL OF and 17±0.5 µL UF per reproductive system were obtained; osmolarity was 343±20.8 mOsm kg^{-1} and 280±96.2 mOsm kg^{-1} and protein concentration was 71.9±23.8 g L^{-1} and 21.8±1.1 g L^{-1}, respectively. In the protein distribution pattern, 20 bands were observed in the OF and 14 bands in the UF. Of these, 14 and 8 were specific for OF and UF, respectively, and 6 were common for both. The spectra of the protein molecular weights were 24–324 and 29–353 kDa for OF and UF, respectively. The presence of OVPG1, HSP70 and Ezrin proteins in both fluids was identified, being in greater quantity in the OF ($P < 0.0005$). The volume recovered from the UF was five times greater than that of the OF. Both osmolarity and protein concentration were higher in OF than in UF (1.2 and 3 times higher). The pattern of protein distribution between the OF and UF was different, being more complex in the OF. OVGP1, HSP70 and ezrin were identified in the OF and UF, and were found in greater quantities in the OF.

Keywords: OVPG1, HSP70, reproductive fluids, osmolarity, SDS-PAGE.

INTRODUCTION

The oviduct is divided into five anatomical regions: infundibulum, ampulla, isthmus-ampullar junction, isthmus, and uterus-tubal junction. Each region participates in gamete maturation, sperm capacitation, fertilization, and the beginning of preimplantation embryonic development (Kölle *et al*., 2020), which ends in the uterus and leads to the implantation process and the beginning of fetal development (Bhusane *et al*., 2016).

The oviductal epithelium and luminal and glandular epithelia from the endometrium are responsible for regulating the microenvironment of each oviductal segment and uterus, formed by populations of secretory and ciliary cells, whose proportion and function depend on the stage of the female estrous cycle or pregnancy (Restall, 1966a; Bhusane *et al*., 2016). Secretory cells synthesize products that are discharged into the lumen and, together with the transudate of the circulatory system, form the oviductal (OF) and uterine fluids (UF), complex mixtures of ions and macromolecules dissolved in water (Bhusane *et al*., 2016; Li & Winuthayanon, 2017).

Sheep embryos remain in the oviduct for an average of three days from the developmental stage of the zygote to the 8 and 16-cells stage. In the uterus, they reach the blastocyst stage by days 6 and 7, the zona pellucida hatching occurs on days 8 and 9 (Abecia & Forcada, 2010), and elongation occurs until day 15. At this point, implantation of the conceptus occurs (Hyttel *et al*., 2010). All of these processes can only be achieved in an environment where nutrients are produced by each structure of the reproductive tract.

Preimplantation embryo development concerning the stages of zygote to blastocyst development can be reproduced under *in vitro* conditions. This requires the use of

synthetic culture medium for embryo development, formulated based on the biochemical composition of the oviductal and uterine fluid (Tervit *et al*., 1972), which corresponds to the early luteal phase of the estrous cycle (Restall & Wales, 1966; Restall, 1966b; Wales, 1973).

Although this type of medium has been widely used, it is known that embryos produced *in vitro* develop in a minor percentage and quality compared to those produced *in vivo*, and they present dysregulation in their genetic expression and an altered pattern of epigenetic marks (Rabaglino *et al*., 2021). Therefore, this condition can be reversed when OF and UF are added to the culture medium (Barrera *et al*., 2017; Canovas *et al*., 2017; Lopera-Vasquez *et al*., 2017; Hamdi *et al*., 2018) as supplements that can supply molecules needed for the adequate development of embryos, such as certain proteins.

Proteomics has been used to describe all proteins expressed in a genome (Wilkins *et al*., 1996), by which proteins from different organisms have been identified, quantified, and separated through techniques ranging from western blotting and ELISA to more sophisticated and complex techniques such as chromatography and mass spectrometry (Aslam *et al*., 2017).

The proteome of reproductive fluids has been analyzed in different domestic species, focusing mainly on hormonal regulation at different stages of the estrous cycle, gamete-maternal interaction, embryonic-maternal communication, follicular development, and fertility (Itze-Mayrhofer & Brem, 2020).

However, in the sheep there are few studies related to the proteome of OF and UF, of which most have focused on determining the main proteins present in UF during the first days of pregnancy, but few have reported about OF (Köch *et al*., 2010; Burns *et al*., 2014; Romero *et al.* 2017). Only one study has determined the proteome of the cervical, oviductal, and uterine fluids during the estrous and late luteal phases of the estrous cycle in sheep, whether spontaneous or induced (Soleilhavoup *et al*., 2016). This study identified the main proteins present in OF and UF, including oviduct-specific glycoprotein (OVGP1), heat shock protein 70 (HSP70), and ezrin. Therefore, studies of proteomes in the early luteal phase could be of great interest as early embryo development occurs.

Thus, the objective of the present study was to determine the physicochemical characteristics (volume, osmolarity, and total protein concentration) and protein profile (distribution pattern of proteins) of oviductal and uterine fluids from domestic sheep, as well as to identify OVGP1, HSP70, and ezrin in OF and UF, in the early and middle luteal phases of the estrous cycle in sheep.

MATERIAL AND METHODS

Collecting of OF and UF

OF and UF were obtained from the reproductive systems of adult criollo-type domestic slaughtered sheep. The reproductive systems were obtained immediately after opening the abdominal cavity and were transported at 8 °C in disinfected plastic bags. Once in the laboratory, within no more than 2 h, the reproductive systems were washed in sterile Dulbecco's phosphate-buffered saline (DPBS) with 1% antibiotic-antifungal (streptomycin 10,000 µg mL^{-1}, Amphotericin B 25 µg mL^{-1}, and 10,000 IU penicillin, *in vitro* S.A. de C.V.) at 4 °C.

Subsequently, they were classified based on the ovarian structures present (to determine the stage of the estrous cycle) (Senger, 2005) with regular size and shape. The luteal phase was divided into the early and middle luteal phases. The early luteal phase was determined by the presence of corpus hemorrhagicum, whereas the middle luteal phase was determined by the presence of corpus luteum (Senger, 2005). Thirty-two reproductive systems were in the early luteal phase and ten in the middle luteal phase.

Fluids were recovered from the ipsilateral oviduct and uterine horn of the ovary in the presence of corpus hemorrhagicum and corpus luteum. Once the reproductive systems were classified, the suspensory ligaments were dissected and the uterus-tubal junction was severed to separate the oviduct from the uterine horn. Oviducts and uterine horns were kept in sterile DPBS at 4 °C.

The method described for cattle was followed for OF extraction (Carrasco *et al*., 2008), with some modifications. On a flat surface, the oviduct was placed and squeezed with a slide from the uterus-tubal junction toward the ampulla, and a clamp was placed to prevent the return of fluid. A fire-polished Pasteur pipette was then introduced to recover the OF and was deposited in a 300 µL tube. The sample was centrifuged at 2000 x g for 5 min at 4 °C, and the supernatant was recovered and centrifuged at 7000 x g for 10 min at 4 °C.

Finally, the supernatant was recovered and stored at -80 °C, until use. To obtain the UF, the reproductive system was placed vertically so that the fluid descended by gravity to the uterus-tubal junction, after which a polished Pasteur pipette was introduced, and the fluid was collected. It was placed in a 300 µL tube, processed, and stored in the same way as the OF. For each experiment, a pull of OF from the early luteal phase and a pull of UF from the middle luteal phase were applied (Carrasco *et al*., 2008). Subsequently, the volume of each fluid was determined. The OF and UF samples were collected from January to May 2022 (winter-spring), which corresponds to the reproductive season in sheep, and then stored for 8 months at -80 °C. After thawing, the physicochemical characteristics were determined for each fluid (osmolarity, total protein concentration, and OVGP1, HSP70, Ezrin proteins).

Determination of physicochemical characteristics of OF and UF

Osmolarity. Eight months after the samples were collected, the physicochemical characteristics of OF and UF were determined. Osmolarity was determined by placing 10 µL of each sample (OF and UF) in an osmometer (Wescor model VAPRO-5520) (Pensyl & Benjamin, 1999).

Total protein. Total protein concentration was determined using the Bradford method. A calibration curve with bovine serum albumin (BSA) was used as a protein standard with a range of 0 to 1 mg mL^{-1}, 500 µL of Bradford solution (100 mg of Coomassie blue G-250 dissolved in 200 mL of 95% alcohol and 85% phosphoric acid), and 10 µL of each BSA concentration in a 1.5 mL tube, in duplicate. To analyze OF and UF, 500 µL of Bradford solution and 1 µL OF or UF were placed in 1.5 mL tubes, in duplicate. Finally, the calibration curve, as well as the OF and UF samples, were analyzed using a spectrophotometer at 595 nm, and the values obtained to determine the total protein concentration of reproductive fluids were recorded (Bradford, 1976).

Distribution pattern of proteins in reproductive fluids (OF and UF)

Protein separation by SDS-PAGE. Protein separation by molecular weight was performed using electrophoresis, and a 10% polyacrylamide gel was prepared. First, the separator gel was prepared using water, tris-hydroxymethyl-aminomethane (TRIS)-HCl (1.5 M, pH 8.8), bis-acrylamide (30%), Sodium Dodecyl Sulfate (SDS, 10%), ammonium persulfate (10%), and tetramethylethylenediamine (TEMED) and allowed to polymerize. The concentrator gel was then added with the same reagents and only the TRIS-HCl concentration was changed (0.5 M, pH 6.8). Buffer Laemmli was placed with 50 mg mL^{-1} of protein from each sample of OF and UF for protein denaturing. The samples were then placed in each well of the polyacrylamide gel, together with a molecular weight marker of 10–250 kDa. The proteins were compacted at 150 V for 15 min and then left to run at 100 V for 180 min (Brunelle & Green, 2014).

Protein identification by molecular weight. After electrophoresis, the gel was stained with a solution of Coomassie blue (0.1% Coomassie blue, 50% methanol, and 10% glacial acetic acid) for 1 h under constant oscillation. Excess stain was removed using a 40% methanol solution for 15 min during oscillation. Several washes with a new methanol solution were performed until bands were visible in the gel (Brunelle & Green, 2014).

Estimation of molecular weight and band intensity. Images of the gels were taken using a photodocumentary. For image processing, Gelanalyzer (version 19.1; Istvan Lazar Jr.) was used to identify the number of bands in each of the OF and UF samples, as well as the estimation of the molecular weight (kDa) of each of the bands.

Identification of proteins in OF and UF

Western blot. To determine the presence of oviduct-specific glycoprotein (OVGP1), heat shock protein 70 (HSP70) and ezrin which are among the most abundant proteins in these fluids (Soleilhavoup *et al.*, 2016), of each of the OF and UF samples, 50 µL mL^{-1} protein was placed in load buffer and incubated at 95 °C for 5 min. Samples were separated on 10% polyacrylamide gels at 120 volts for 60 min. The proteins were then transferred to a PVDF membrane (Millipore, VH00010) at 2.5 A and 25 V for 20 min on a transfer device (Trans-Blot Turbo).

The membrane was blocked with TBS buffer milk solution (0.08 g mL^{-1}) for 1 h, in constant oscillation at room temperature. The membrane was then incubated with the first antibody (Ezrin: Cat. Sc-58758; HSP70: Cat. sc-66048; OVGP1: Cat. sc-377267, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) diluted in a milk solution with TBS (0.05 g mL^{-1}) in constant oscillation all night at 4 °C. The next day, the membrane was washed with TBS Tween-20 and incubated with the secondary antibody (Anti-mouse IgG: Cat. 715-035-150; Jackson ImmunoResearch, West Grove, PA, USA) for 1.5 h oscillating at room temperature. The membrane was then washed with TBS Tween-20. Finally, a mixture of revealing solution (ClarityTM Western ECL Substrate, Bio-Rad) was added, and the membrane was placed in a photo documenter (Kodak Gel Logic 200 Image System), with a 3 min exposure for its development. The program used was the Kodak 1D 3.6 Logic.

ImageJ software was used for image processing. This program measures the intensity of the pixels in an image. This is especially useful in Western Blot images, where the signal intensity can be correlated with the amount of a certain protein. To do this, the program selects the Region of Interest (ROI) around a specific region of the image in which the analysis needs pixel intensity. This generated a graph that shows the pixel intensity of the ROI. ImageJ provides numerical values associated with the ROI, from which statistical analysis will be performed.

Statistical Analysis

The data obtained on volume, osmolarity, and total protein concentration of OF and UF samples are expressed as the mean ± standard deviation (S.D.). To determine significant differences between the color intensities OF and UF sample bands, Student's t-test was performed with a significance of $P < 0.05$. GraphPad Prism (version 9.5.1) was used for data analysis.

RESULTS

OF and UF were obtained from 42 reproductive systems of domestic sheep. Of these, 32 were in the early luteal phase and 10 were in the middle luteal phase. A pool of OF (2-7 reproductive systems) and UF (1-6 reproductive systems) was prepared for each experiment, and a total of 10 OF samples and 3 UF samples were obtained.

Physicochemical characteristics of OF and UF

Volume, osmolarity, and total protein concentration of the reproductive fluids (OF and UF). A total volume of 135 µL (3.2±1.5 µL on average) of OF from the early luteal phase and 170 µL (17.0±0.5 µL) from the middle luteal phase were recovered from the reproductive apparatus at the luteal phases of the estrous cycle in sheep. The osmolarity for OF was 343±20.8 mOsm kg^{-1}, and that for UF was 280±96.2

mOsm kg^{-1}. The total protein concentration of OF was 71.9±23.8 g L^{-1}, while that of UF was 21.8±1.1 g L^{-1}.

Distribution patterns of proteins in the reproductive fluids (OF and UF). Figure 1 shows the pattern of protein distribution in 10 OF and 2 UF samples. The molecular weights of the protein bands were 24–324 kDa for OF and 29–353 kDa for UF.

In the analysis of the OF and UF samples, two sets of bands were identified, with 20 bands present in the OF and 14 bands present in the UF. Of these, fourteen bands were unique to the OF samples, while eight were exclusive to the UF samples. Furthermore, six bands were identified as being present in both the OF and UF samples (as shown in Figure 1 and detailed in Table 1).

Identification of proteins in OF and UF. In the distribution pattern of OF and UF proteins (Figure 1), different types of proteins are shown, some of which were identified by western blotting, where OVGP1 (120 kDa), HSP70 (70 kDa), and ezrin (87 kDa) proteins were present in most OF and UF (Figure 2a). However, the levels of these proteins were significantly higher in the OF than in the UF (Figure 2b).

Table 1.
Molecular weight of proteins present in OF and UF samples.

No. of band	OF specific MW (kDa)	UF specific MW (kDa)	In common MW (kDa)
1	324	353	73
2	315	284	37
3	306	138	34
4	228	111	28
5	200	47	27
6	188	42	26
7	83	39	
8	62	29	
9	54		
10	36		
11	35		
12	33		
13	32		
14	24		

OF, Oviductal fluid; UF, Uterine fluid; MW, Molecular weight; kDa, kiloDaltons

DISCUSSION

Physicochemical characteristics of OF and UF. Two pools were created in this study. One was of OF from the early luteal phase and the other was a pool of UF from the middle luteal phase because embryos are in each one of these structures as luteal phases occur (Kölle *et al*., 2020). OF and UF were collected from *postmortem* adult Criollo type domestic sheep, with a volume of UF five times greater than OF, which may be related to the size of the organs of origin. In other studies, OF and UF volumes were collected from sheep *in vivo* and analyzed continuously for 24 h using a probe introduced into the ostium of the oviduct. The authors reported higher volumes of OF and UF compared to those obtained in our study, with 100 to 1,630 µL for OF (Restall, 1966b; Perkins *et al*., 1965; Iritani *et al*., 1969; Roberts *et al*., 1976) and 480 to 5,210 µL for UF (Iritani *et al*., 1969). These volumes account for the intense secretory activity of the oviduct and uterus, which may be influenced by the probing recovery system, is also influenced by hormonal action. It has been observed that 2 days after estrous, both the oviduct and uterus present greater secretory activity (Iritani *et al*., 1969), which confirms that there are factors related to the modulation of the secretory pattern of the fluids in these anatomical regions. In addition, other factors influence the secreted amount of OF and UF, for example, such as age, breed, and whether the females are prepubertal, nulliparous, or multiparous.

Another important physiological aspect to consider is the osmolarity of OF and UF, which depends on the concentration of dissolved solutes, such as inorganic salts, amino acids, and proteins, among other components, which are in direct contact with gametes and developing embryos that require specific osmolarity conditions. The range of variation of oviductal and uterine osmolarity between different domestic species is narrow; for example, in cows, an

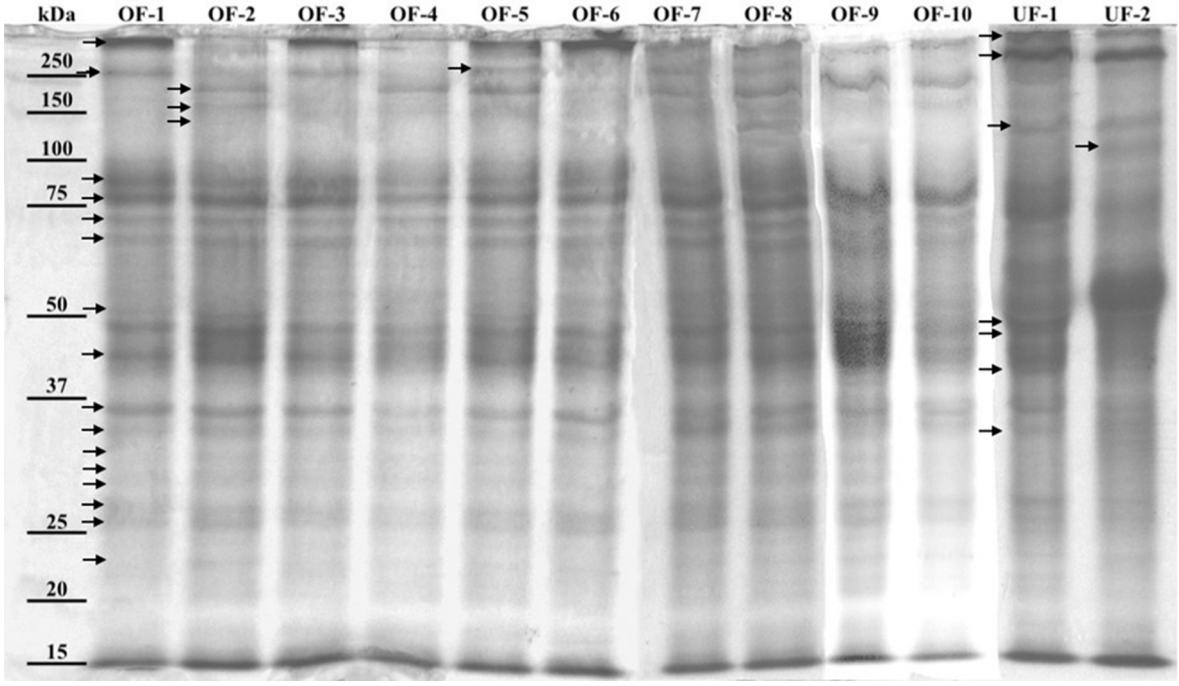

Figure 1.
The allocation of proteins in the sheep oviductal fluids (OF-1 to OF-10) and uterine fluids (UF-1 and UF-2) was assessed through SDS-PAGE. Fourteen bands where unique to OF samples, eight were exclusive to UF samples, and six were present in both samples. Specific molecular weights of proteins are indicated in Table 1.

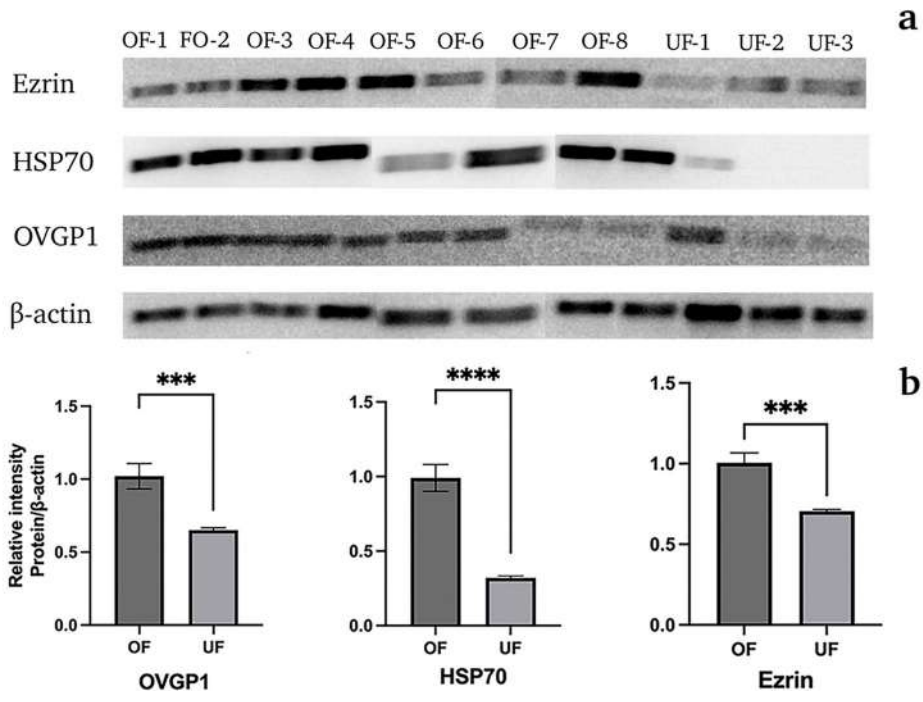

Figure 2.
The presence of OVGP1, HSP70, and ezrin proteins was examined in the OF and UF samples from sheep using Western blotting. β-actin was used as a positive control (a). The concentration of OVGP1, HSP70, and ezrin was then assessed between the OF and UF samples, revealing a statistically significant difference between the two groups (P = 0.0005 and P = 0.0001, respectively) (b).

osmolarity of 350–353 mOsm kg^{-1} has been reported (Olds & Vandemark., 1957), in sows it is 318-320 mOsm kg^{-1} (Li *et al*., 2007), and in sheep, an osmolarity of 316-349 mOsm kg^{-1} has been reported for OF and UF (Wales, 1973). These values are similar to those observed in this study for OF (343 mOsm kg^{-1}) but lower than that observed in UF (280 mOsm kg^{-1}). This variation in UF osmolarity was probably due to manipulation during the collection and processing of samples; for example, it has been reported that both the method and collection site influence the osmolarity of UF (Wales, 1973).

The total protein concentration present in OF was higher (71.9 g L^{-1}) than that reported in other studies with concentrations of 37.8 and 38.7 g L^{-1} (Itze-Mayrhofer & Brem, 2020; Zhao *et al*., 2022). The total UF protein concentration was similar (21.8 g L^{-1}) to that reported by other authors (25.16 to 27.46 g L^{-1}) (Tripathi *et al*., 2016; Yahia *et al*., 2013). The increase in total OF protein concentration could be due to metabolic stress conditions derived from the diet (Tripathi *et al*., 2016).

In a first evaluation, the distribution pattern of the proteins present in OF (20 bands) and UF (14 bands) was determined, as well as the molecular weights of the bands with greater intensity, as has been done in Alpaca (Apichela *et al*., 2015). From this first count, differences in the distribution pattern of the proteins present between the OF and UF samples were observed, a greater number of bands for OF being observed, which denotes a greater complexity in their protein composition, However, studies have identified more proteins in UF than in OF (827 vs. 624), a fraction of these proteins (585) occur in both fluids (Soleilhavoup *et al*., 2016).

Identification of proteins in OF and UF. Subsequently, three proteins were identified using western blotting: OVGP1, HSP70, and ezrin. OVGP1 is a protein synthesized and secreted exclusively by the non-ciliary cells of the oviductal epithelium, with a molecular weight between 90 and 95 kDa for most domestic animals; in ruminants, this is in the range of 57.23–57.75 kDa, which is attributed to the degree of glycosylation of the protein (Pradeep *et al*., 2011; Zhao *et al*., 2022).

Moreover, HSP70 belongs to a family of "chaperones" proteins with a molecular weight of 70 kDa that perform a wide variety of cell maintenance activities, as well as to counteract the effects caused by stress, such as: preventing protein aggregation, the separation of protein aggregates, the replication of denatured proteins and the degradation of defective proteins (Rosenzweig *et al*., 2019).

Ezrin is an protein of 87 kDa molecular weight that functions as a microfilament connector of the plasma membrane, which is distributed in the microvilli, folds of the plasma membrane, and other areas of this membrane, with a particular morphology (Xu *et al*., 2023).

In the present study, OVGP1 was found in greater numbers in OF samples, which is in accordance with Soleilhavoup *et al.* (2016), who evaluated the OF and UF proteome in estrous and during the luteal phase of the sheep estrous cycle, by nanoscale liquid chromatography coupled to tandem mass spectrometry. The authors determined that OVGP1 was the main OF protein during estrous. In contrast, OVGP1 was present in smaller quantities in UF, which corresponds to what was reported for UF (Soleilhavoup *et al*., 2016). However, extracellular vesicles obtained from the uterine lumen of sheep have been described as positive for OVGP1 (Burns *et al*., 2014).

OVGP1 plays a significant role during fertilization because it regulates polyspermy by hardening the pellucida zone (Bragança *et al*., 2021). It has also been reported in goats that morulae and blastocysts rate increases (Pradeep *et al*., 2011). Immunohistochemistry has detected the interaction of OVGP1 with bovine embryos in stages of 4-8 cells and in morulae, in the perivitelline space, and within blastomeres, but not in the pellucida zone (Banliat *et al*., 2020).

The presence of HSP70 protein was observed in the OF, but it was practically absent in the UF, which coincides with what has been reported in sheep (Soleilhavoup *et al*., 2016). However, others have identified it in the UF on day 16 of the estrous cycle, but not in sheep on day 16 of pregnancy (Köch *et al*., 2010). Microvesicles of the uterine lumen are positive for HSP70 on day 14 in both cycling and pregnant sheep (Burns *et al*., 2014).

As mentioned, HSP70 performs activities to counteract the effects of stress. Under suboptimal temperatures of *in vitro* culture (37 and 40 °C), HSP70 is overexpressed in both oocytes and granulosa cells (Pöhland *et al*., 2020). This has also been reported in cattle, where inhibition of HSP70 function during culture of 2-cell embryos at physiological temperature (38.5 °C) reduces the percentage of blastocysts. This shows the importance of HSP70 in cellular functions, not only in relation to caloric stress (Romero & Hansen, 2002).

Finally, ezrin protein was found in greater quantities in OF than in UF, which is consistent with the results reported for sheep (Soleilhavoup *et al*., 2016). It has also been reported in the UF of sheep during the first days of pregnancy (Köch *et al*., 2010; Romero *et al*., 2017).

Xu *et al.* (2023) reviewed the function of ezrin protein from a reproductive perspective, mentioning its importance during changes that occur in the uterine epithelium throughout the estrous or menstrual cycle. Ezrin induces polarization of preimplantation embryos as well as processes of cellular migration and invasion during embryo implantation and embryogenesis.

The volume recovered from UF was five times greater than that recovered from OF, which is related to the size and surface area of the oviduct and uterus. Both osmolarity and protein concentration were higher in OF than in UF (1.2 and 3 times higher, respectively), values that were within the range described for sheep. The pattern of protein distribution between the OF and UF was different, being more complex in the OF. OVGP1, HSP70, and ezrin were identified in OF and UF, and were found in greater numbers in OF samples.

CONCLUSIONS

The present study determined the physicochemical characteristics (volume, osmolarity, and total protein concentration), protein profile (distribution pattern of proteins), and the presence of OVGP1, HSP70, and ezrin proteins in the early and middle luteal phases of the estrous cycle of adult domestic sheep to determine its usefulness as a supplement to optimize *in vitro* culture media for embryo development by providing elements lacking commercial growing media.

Competing interest's statement

The authors declare that they have no conflicts of interest.

Author contribution

Conception: D.A.A.G., G.B.F., M.A.F.B. and J.R.V.A. Execution of experiments: J.R.V.A., H.L.R., M.A.F.B., and M.P.C.O. Data analysis and interpretation: M.A.F.B., D.A.A.G., and J.R.V.A. Original draft: J.R.V.A. Revisions: D.A.A.G., C.C.R., G.B.F., M.A.F.B. and M.C.N.M. Disposition of funds, materials, and equipment: D.A.A.G., G.B.F., and M.C.N.M.

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ORIGINAL ARTICLE

Prevalence, risk factors, and hematologic changes in dogs from Baja California with presence of *Ehrlichia* spp., and coinfection with *Anaplasma* spp.

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ABSTRACT. *Ehrlichia* and *Anaplasma* are obligate intracellular, gram-negative bacteria with tropism for hematopoietic cells, especially leukocytes and platelets. There are several *Ehrlichia* species that infect dogs. *Ehrlichia canis* is transmitted by *Rhipicephalus sanguineus* and replicates within monocytes and macrophages, leading to canine monocytic ehrlichiosis, a disease of worldwide distribution. The clinical signs are varied and non-specific. *Anaplasma* has two species that infect dogs, *Anaplasma phagocytophilum* and *Anaplasma platys*, the second also transmitted by *Rhipicephalus sanguineus*. This study aimed to evaluate the epidemiology and hematologic changes associated with the presence of *Ehrlichia* spp. and *Anaplasma* spp. coinfection in dogs from Baja California. Complete hematological analysis, examination of buffy coat smears, and ELISA tests were performed on blood samples from three veterinary diagnostic laboratories from Mexicali and Tijuana cities in Baja California, Mexico. A total of 5.469 dog samples were analyzed. The overall prevalence of *Ehrlichia* spp., was 4.79%, with a distribution of 6.3% in Mexicali (OR: 2.39 CI: 1.69-3.17) and 2.5% in Tijuana. The peak of infection was found in September. Contact with other dogs and tick infestation were the risk factors associated with infection ($P < 0.05$). There was 96% co-infection only in Tijuana and 0% in Mexicali. Anemia, thrombocytopenia, and hyperproteinemia are associated with *Ehrlichia* spp., and *Ehrlichia/Anaplasma* infection. In view of the foregoing, we have to maintain epidemiologic vigilance, as well as look further into the ticks present in the state and the possibility of transmission of unusual pathogens.

Keywords: *Ehrlichia*, *Anaplasma*, coinfection, prevalence, risk factors, hematologic changes.

INTRODUCTION

There are many pathogens transmitted by vectors that affect dogs. *Ehrlichia* is a genus of obligate intracellular, gram-negative bacteria in the family *Anaplasma*taceae, order Rickettsiales (Ramakant *et al*., 2020), with tropism for hematopoietic cells, especially leukocytes and platelets (Dhavalgi *et al*., 2021). Several *Ehrlichia* species infect dogs, including *E. ewingii*, *E. chaffeensis*, *E. muris*, and *E. canis*. *Ehrlichia ewingii* is found more often in neutrophils and *E. chaffeensis* in monocytic cells, both of which are transmitted by the lone star tick (*Amblyomma americanum*) (Lashnits *et al*., 2019; Xu *et al*., 2023). *Ehrlichia muris* also infects monocytic cells (Feng & Walker, 2004), but is transmitted by black-legged ticks (*Ixodes scapularis*) (Xu *et al*., 2023). *Ehrlichia canis* is transmitted by the brown dog tick (*Rhipicephalus sanguineus*) and replicates within monocytes and macrophages (Rikihisa, 2021), leading to canine monocytic ehrlichiosis (CME), a disease with a worldwide distribution (Christodoulou *et al*., 2023). Canine monocytic ehrlichiosis has been reported in Asia (Ansari-Mood *et al*., 2010; Bhadesiya & Modi, 2015; Kottadamane *et al*., 2017; Mittal *et al*., 2017; Haryanto & Tjahajati 2020), Europe (Pantchev *et al*., 2015; Sainz *et al*., 2015; Piantedosi *et al*., 2017; Jurković *et al*., 2019), and the Americas (Carrade *et al*., 2011; Melo *et al*., 2011; Villeneuve *et al*., 2011; Barrantes-González *et al*., 2016; Pesapane *et al*., 2019).

In Mexico, the seroprevalence of *E. canis* ranges from 33.1% to 74.3% (Sosa-Gutiérrez *et al*., 2013; Salinas-Meléndez *et al*., 2015; Almazán *et al*., 2016; Movilla *et al*., 2016), however, epidemiological studies on this disease in canine populations in the State of Baja California are insufficient. Haro-Álvarez *et al*. (2007) reported a seroprevalence of 21.6% (83/384) in dogs treated at veterinary clinics, including only dogs suspected of having the disease in the city of Mexicali. However, the prevalence of this disease in other cities in the state is unknown. Worldwide, various risk factors have been associated with CME, including age (Pinter *et al*., 2008; Vieira *et al*., 2013; Milanjeet *et al*., 2014), seasonality (Lee *et al*., 2020), presence of ticks in dogs (Yuasa *et al*., 2012; Huerto-Medina & Damasco-Mata 2015; Navarrete *et al*., 2018), and lack of veterinary care (Pérez-Macchi *et al*., 2019), among others.

Anaplasma is an obligate intracellular gram-negative bacterium from the same family and order as *Ehrlichia*, with worldwide distribution (Rar *et al*., 2021). Two species of *Anaplasma* infect dogs, *A. phagocytophilum* and *A. platys*. The first is re-

sponsible for canine granulocytic anaplasmosis (CGA) as it infects granulocytes and is transmitted by *Ixodes* ticks (Carrade *et al*., 2009). In Mexico, it has been reported in various states, but principally in the northern states of the country (Aragón-López *et al*., 2021), however, it has not been reported in Baja California. The second is the causative agent of infectious canine cyclic thrombocytopenia (ICCT) as it infects platelets, is transmitted by *R. sanguineus* (Atif *et al*., 2021) and regarding Mexico, it has been reported by PCR in dogs from Cajeme, Sonora, with 10.58% of prevalence (Aragón-López *et al*., 2021), 31% from the region known as "La Comarca Lagunera" (Almazán *et al*., 2016) and 24.74% in Ciudad Juarez, Chihuahua (Beristain-Ruiz *et al*., 2022). There are also reports of antibodies to *Anaplasma* spp. in several states of Mexico, including Baja California, with a seroprevalence of 32.9% (Bedoya *et al*., 2023).

Considering the limited knowledge about the epidemiology of CME in the region, the objective of this study was to estimate the prevalence, risk factors, and hematological changes associated with CME in dogs with owners in two cities in northwest Mexico and to search for antibodies against *Anaplasma* spp., since both diseases are endemic (Aragón-López *et al*., 2021).

MATERIAL AND METHODS

Data were obtained from 5,469 blood samples from dogs remitted to three veterinary diagnostic laboratories in the cities of Mexicali and Tijuana, Baja California, Mexico, between September 2021 and August 2022. Blood samples (0.5 mL) with EDTA and non-hemolyzed plasma from dogs aged one month of age or older, of any breed, size, and sex were included. Each sample received was processed for complete hematological analysis that included the measurement of hematocrit and total solids and the counts of erythrocytes, leukocytes, and platelets. Blood smears were analyzed to check the leukocyte differential and cell morphology, as well as to search for intracellular morulae of *Ehrlichia* and the presence of structures compatible with *Anaplasma*. Buffy coat smears were performed for every patient to maximize the possibility of finding morulae.

Serology

When no morulae were found, but there was clinical and hematological suspicion of ehrlichiosis, ELISA tests were performed. Some samples were originally sent from veterinary clinics for complete hematological studies and ELISA tests. The ELISA tests utilized (IDEXX® Snap 4DX plus) detects the presence of antibodies against *E. canis/E. ewingii*, *Borrelia burgdorferi*, *Anaplasma platys/A. phagocytophilum* and *Dirofilaria immitis*, with 97.6% sensitivity and 99.0% specificity, as well as IgG antibodies, particularly for *Ehrlichia* spp. and *Anaplasma* spp. (Kaewmongkol *et al*., 2020; Zhang *et al*., 2022).

Cases were considered *Ehrlichia*-positive based on the presence of *Ehrlichia* morulae in the blood/buffy coat smear or by a positive ELISA result. Cases were considered *Anaplasma*-positive only by ELISA tests, as there was no presence in any blood smear.

Data Collection to determine risk factors

In order to determine the risk factors associated with infection, age (1-12 months or >12 months), sex (male or female), breed (mixed or pure), city of origin (Mexicali or Tijuana), presence of ticks (yes or no), street access (yes or no), and contact with other dogs in the house (yes or no) were registered.

Statistics

The prevalence of the disease was determined by the number of positive cases and the number of patients attended by year. Chi-square (χ^2) estimation, *P*-values and odds ratio with 95% confidence interval were calculated for the association between risk factors and the disease. Furthermore, risk factors with $P < 0.10$ were analyzed using a binomial logistic regression model. The dependent variables were positive *Ehrlichia* cases, and the independent variables were the risk factors described above. To identify statistical differences between hematologic alterations in 1) positive and negative *Ehrlichia* patients; 2) positive for *Ehrlichia* patients by the presence of morulae and positive by ELISA tests; and 3) positive for *Ehrlichia* spp. and positive for *Ehrlichia spp*. and *Anaplasma* spp., Student's t-tests were performed. Probability values less than 0.05 ($P \leq 0.05$) were considered statistically significant. Inferential analysis was performed using Statistix 9® software.

RESULTS

A total of 5,469 dog samples were analyzed. The overall prevalence of *Ehrlichia* spp. was 4.79% (262/5,469), and its distribution among cities was 6.3% (206/3,269) in Mexicali and 2.5% (56/2,200) in Tijuana. Of all the positive cases, 149 were positive for the presence of morulae within mononuclear cells and 105 were positive by ELISA. Eight samples tested positive using both diagnostic methods. Dogs in Mexicali had a 2.57 times higher risk of infection than those in Tijuana. The presence of ticks showed 1.78 times higher likelihood of having the disease, and dogs that had contact with other dogs had a 1.86 times higher risk of being affected by *Ehrlichia* spp. The other evaluated variables were not statistically significant (Table 1).

In the multivariate analysis, the only risk factor associated with *Ehrlichia* infection was the origin of the dog (Table 2), which was 2.31 times more likely to find dogs positive for *Ehrlichia* spp. in Mexicali than in Tijuana. The other factors analyzed were not statistically significant.

The monthly/seasonal tendency of *Ehrlichia* cases was higher in Mexicali (Figure 1). In every city, the number of infected animals peaked in September. In Mexicali, another outbreak occurred in June and July, with both cities having a higher frequency during summer.

In this study, 96% (54/56) of the *Ehrlichia* spp. cases identified in Tijuana showed antibodies against *Anaplasma* spp. by ELISA. Notably, all cases of coinfection were found in Tijuana.

To evaluate the behavior of some hematological indicators between *Ehrlichia* spp., positive and negative cases, we compared the mean values for hematocrit, neutrophils, lymphocytes, platelets, and total solids. The results showed statistically significant differences ($P < 0.05$) between all variables mentioned above, except for neutrophils. However, in cases positive by ELISA, neutrophilia was found, and in every group, we found occasional Döhle bodies and diffuse basophilia (Table 3).

In Table 4, we show the differences found in blood analytes between patients positive for *Ehrlichia* morulae and those positive by ELISA tests. There was statistical difference in lymphocyte and platelet counts.

In Table 5, we present the differences in blood analytes between patients positive for *Ehrlichia* spp. and *Ehrlichia*/*Anaplasma* spp. There was statistical difference in platelets and total solids.

Table 1.
Risk factors associated with the presence of Ehrlichia spp. in dog samples from Baja California.

	N	Positives (%)	OR	95% CI	*P*
City					
Mexicali	3,269	206 (6.3)	2.57	1.91 – 3.47	0.00*
Tijuana	2,200	56 (2.5)			
Access to Street					
Yes	109	8 (7.3)	1.59	0.77 – 3.31	0.21
No	5,360	254 (4.7)			
Presence of ticks					
Yes	290	23 (7.9)	1.78	1.14 – 2.78	0.01*
No	5,179	239 (4.6)			
Contact with other dogs					
Yes	744	57 (7.6)	1.86	1.37 – 2.52	0.00*
No	4,679	200 (4.2)			
Sex					
Female	2,911	141 (4.8)	1.04	0.80 – 1.34	0.78
Male	2,414	113 (4.6)			
Age					
0-12 months	1,062	57 (5.3)	1.16	0.86 – 1.57	0.32
>12 months	4,406	205 (4.6)			

CI: Confidence interval; OR: Odds ratio; P: probability value; * P < 0.05

Table 2.
Risk factors associated with the presence of Ehrlichia spp. in dogs in Baja California. Multivariate analysis.

	N	Positives (%)	OR	95% CI	*P*
City					
Mexicali	3,269	206 (6.3)	2.31	1.69 – 3.17	0.00*
Tijuana	2,200	56 (2.5)			
Presence of ticks					
Yes	290	23 (7.9)	1.34	0.85 – 2.10	0.21
No	5,179	239 (4.6)			
Contact with other dogs					
Yes	744	57 (7.6)	1.33	0.96 – 1.83	0.08
No	4,679	200 (4.2)			

CI: Confidence interval; OR: Odds ratio; P: probability value; * P < 0.05

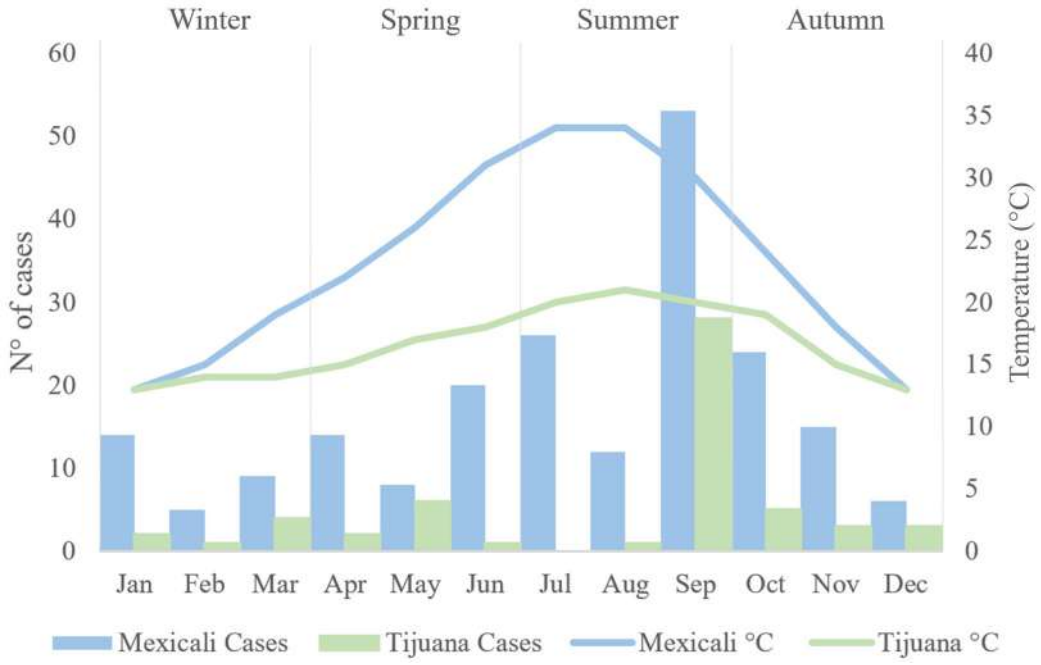

Figure 1.
Monthly and seasonal behavior of positive cases to Ehrlichia spp., in dogs from the cities of Mexicali and Tijuana, Baja California, México. We also show the average temperature in every city.

Table 3.
Hematologic alterations in positive patients by presence of Ehrlichia spp., morulae, or by ELISA test, and negative for Ehrlichia spp.

	Positive cases		Negative cases			
	Average	**Range**	**Average**	**Range**	***P***	**Ref. Int.**
Ht L/L	0.35±0.10	0.08-0.65	0.41±0.10	0.02-0.68	0.00*	0.37-0.55
N x10⁹/L	10.5±16.7	0.0-244.0	10.1±9.2	0.0-150.0	0.57	3.0-11.5
L x10⁹/L	1.8±1.9	0.0-12.0	2.1±1.9	0.0-33.8	0.02*	1.0-4.8
P x10⁹/L	119±118	8-520	279±149	0-1369	0.00*	200-600
TS g/L	73±17	4-120	76±12	16-120	0.00*	60-75

Ht: Hematocrit; N: Neutrophils; L: Lymphocytes; P: Platelets; TS: Total solids; P: probability value; *P < 0.05; Ref. Int.: Reference Interval (Núñez & Bouda, 2007).

Table 4.
Hematologic alterations in positive cases to Ehrlichia spp., in dogs due to the presence of morulae and by ELISA test.

	Morulae (+)		ELISA (+)			
	Average	Range	Average	Range	*P*	Ref. Int.
Ht L/L	0.36±0.08	0.08-0.56	0.34±0.11	0.08-0.65	0.06	0.37-0.55
N x10⁹/L	9.7±8.6	0.0-78.3	11.7±22.0	0.0-244.0	0.33	3.0-11.5
L x10⁹/L	2.22±2.11	0.1-11.8	1.41±1.49	0.0-12.0	0.00*	1.0-4.8
P x10⁹/L	195±115	28-520	37±40	8-334	0.00*	200-600
TS g/L	4±120	17-72	30±120	18-74	0.43	60-75

Ht: Hematocrit; N: Neutrophils; L: Lymphocytes; P: Platelets; TS: Total solids; P: probability value; *P < 0.05; Ref. Int.: Reference Interval (Núñez & Bouda, 2007).

Table 5.
Hematologic alterations in patients positive to Ehrlichia spp., and positive to Ehrlichia/Anaplasma spp.

	Ehrlichia (+)		*Ehrlichia and Anaplasma* (+)			
	Average	Range	Average	Range	*P*	Ref. Int.
Ht L/L	0.35±0.10	0.08-0.65	0.36±0.09	0.14-0.54	0.70	0.37-0.55
N x10⁹/L	10.8±18.3	0.0-244.0	9.4±8.3	0.9-50.2	0.55	3.0-11.5
L x10⁹/L	1.9±2.0	0.0-12.0	1.4±1.3	0.1-6.0	0.09	1.0-4.8
P x10⁹/L	136±127	8-520	62±48	10-334	0.00*	200-600
TS g/L	72±17	4-120	79±18	50-120	0.00*	60-75

Ht: Hematocrit; N: Neutrophils; L: Lymphocytes; P: Platelets; TS: Total solids; P: probability value; *P < 0.05; Ref. Int.: Reference Interval (Núñez & Bouda 2007).

DISCUSSION

This study represents the first report on the prevalence and distribution of *Ehrlichia* spp. by analyzing a large number of dogs in two cities in the State of Baja California, Mexico.

The only available publications on prevalence rates in the state were conducted by Núñez (2003), who reported a seroprevalence of 70.2% in Baja California. It is important to note that this study only analyzed 37 dogs that attended veterinary clinics with or without compatible signs of the disease, and the specific city where the samples were collected was not specified. Additionally, Haro-Álvarez *et al.* (2007) reported a prevalence of 21.6% in Mexicali. One possible explanation for the difference in prevalence (21.6% vs. 6.3%) could be the rickettsiosis outbreak that occurred in Mexicali in 2009, when several human deaths occurred, leading to increased awareness among pet owners and the Health Department regarding the importance of implementing preventive medicine programs to combat ticks in the municipality. This included programs for junkyard clearance in neighborhoods with a high prevalence and fumigation of homes and pets. Furthermore, the Institute of Research in Veterinary Sciences collaborated through a university rickettsiosis program to provide informative talks to educate the population on these topics.

Recently, Backus *et al.* (2022) conducted a study in four locations in the area (San Diego, Imperial, Tijuana, and Mexicali) from October 2021 to May 2022, analyzing 63 animals in Mexicali and 78 in Tijuana, founding seroprevalences for *Ehrlichia* spp., of 49.2% and 39.7%, respectively. However, the forementioned study is not comparable with ours, since they analyzed abandoned dogs that were kept in confinement, whereas we analyzed samples from owned dogs. Therefore, in the study

by Backus *et al.* (2022), the possibility of transmission and disease was greater than in our study.

In the present study, the prevalence found in Mexicali was 6.3% (206/3269), which is similar to the 7.6% reported in southern Italy (Ebani, 2019) and the 10.0% reported in Iran (Abdous *et al.*, 2024). These results, in the case of Mexicali, are consistent with the fact that vector mortality increases as temperature increases and relative humidity decreases (Tian *et al.*, 2023), as the average temperature in these cities varies from 23.7°C, 14.8°C and 19.6°C respectively, and the mean annual precipitation varies from 0.3, 1.7 and 0.6 inches respectively (Weather Spark, 2024a). In the case of Tijuana, the prevalence observed in our study was 2.5% (56/2200), which is similar to the reported rates of 2.43% in the north central region of Mexico (Aguascalientes, Guanajuato, and Queretaro) (Movilla *et al.*, 2016). Tijuana and these areas have similar temperatures, averaging around 12.7-15.0°C, however, while Tijuana's mean annual precipitation is 0.7 inches, in the other locations vary between 1.3-1.6 inches (Weather Spark, 2024b).

However, our results are very different from those reported by Díaz-Medina *et al.* (2016) in Yucatan, who found a prevalence of 69.2%. Furthermore, these results were obtained using nested PCR in a completely different climate that included an average temperature of 26°C and a relative humidity of 83%, which are favorable conditions for the vectors, promoting their longevity and feeding activity (Abdous *et al.*, 2024). Similar conditions are present in Matto Grosso, Brazil, where Melo *et al.* (2011) reported a 70.9% seroprevalence in dogs from urban and rural areas. Another result was from Ceylan *et al.* (2021), who found a seroprevalence of 19.8% in Turkey, where, although it is not as warm as Yucatan or Brazil, there is a lot of rainfall annually.

It is very relevant that in Baja California, the climatic conditions for the vector life cycle are better in Tijuana (16.6°C and 0.7 inches of rain) (Weather Spark, 2024a) than in Mexicali (23.7°C and 0.3 inches of rain) (Weather Spark 2024b); however, the prevalence of infection in dogs is higher in Mexicali (6.3% versus 2.5%), and outbreaks even occur in the hottest and driest months of the year; therefore, this could be due to heat stress in dogs, as it leads to immunosuppression and thus, the presence of the disease in both the chronic and acute phases (Price *et al.*, 1987; Procajlo *et al.*, 2011). In this regard, we should not underestimate climate change and possible adaptations in vectors, as ecological studies are necessary to understand the natural history of the disease and vector behavior in these extreme climates.

The presence of ticks and cohabitation or contact with other dogs are significant risk factors in animals with the disease, as they are more likely to come into contact with other dogs carrying the vector or places where the vector may be abundant (Yuasa *et al.*, 2012; Huerto-Medina & Dámaso-Mata, 2015; Navarrete *et al.*, 2018). In our univariate analysis, we observed that this risk factor is significant, but this association was not demonstrated in the multivariate analysis, indicating that other conditions in the city of Mexicali play an important role in the presentation of *Ehrlichia*.

The sex and age of the animal did not show statistical significance and can be infected indiscriminately, as reported in other studies (Milanjeet *et al.*, 2014; Navarrete *et al.*, 2018). This could be explained by the presence of ticks and contact with other dogs. If contact occurs at any age or sex, the disease is considered present.

Regarding hematological analyses, we found anemia in every positive group but the positive by morulae group. Mild anemia was observed in every group, but it was significantly different between the negative and positive patients for *Ehrlichia*, which is different from the results obtained by Merino-Charrez *et al.* (2021), who found no statistical difference in anemias between positive and negative *Ehrlichia* patients. This could be explained by the time of infection and the moment of the test, as Gaunt *et al.* (2010) reported that anemia is present in *E. canis* and *E. canis/A. platys*-positive patients from 10-20 days post infection and until 70 days post-infection, after which the hematocrit could be normal again.

In the case of platelets, thrombocytopenia was observed in the mean values of every positive group (table 3-5) being more severe in patients positive by ELISA, probably because of the chronicity of the disease (Gaunt *et al.*, 2010) and *Ehrlichia/Anaplasma* coinfection, since rickettsial organisms commonly produce thrombocytopenia (Chapman *et al.*, 2023) being, in fact, one relevant finding in CME as well as CGA (Khatat *et al.*, 2021) and ICCT (da Silva *et al.*, 2016), since these agents can lead to immune-mediated platelet destruction, consumption due to hemorrhage, and decreased production (Lara *et al.*, 2020). Possible explanations for this behavior include myelosuppressive activity, vasculitis, and immune-mediated destruction in affected individuals, resulting in decreased erythroid and megakaryocytic production in the bone marrow (Martínez *et al.*, 2015; Ybañez *et al.*, 2016).

Few studies have reported that *E. canis* can affect the myeloid cell line, resulting in leukopenia (Martínez *et al.*, 2015; Ybañez *et al.*, 2016; Piratae *et al.*, 2019; Asgarali *et al.*, 2012); however, this effect was not observed. Nevertheless, lymphocytes were significantly lower in positive patients than in negative ones (Table 3), ELISA-positive than in morulae-positive patients (Table 4), and positive for *Ehrlichia/Anaplasma* than in *Ehrlichia*-positive patients (Table 5), possibly as a response to endogenous corticosteroids due to stress (Boes & Durham., 2017), although lymphopenia was found in *Ehrlichia* patients (Bhadesiya & Modi, 2015; Villaescusa *et al.*, 2012; Quorollo *et al.*, 2019) as part of the redistribution during the acute phase response (Long & Vodzak, 2018). It is important to mention that all morulae were found within lymphocytes and monocytes, which is relevant because, although there are reports of *E. canis* morulae within neutrophils (Moura *et al.*, 2019), *E. canis* and *E. chaffeensis* use to produce morulae within mononuclear cells (Aziz *et al.*, 2023). However, *E. chaffeensis* is transmitted by *A. americanum* (Pasternak & Palli 2023), a tick only present in central and eastern Mexico

(Guzmán-Cornejo *et al*., 2011), and the USA (Rochlin *et al*., 2022), whereas *R. sanguineus*, the vector for *E. canis*, is a tick with worldwide distribution and is endemic to Baja California (Sánchez-Montes *et al*., 2021); therefore, it is very likely that the *Ehrlichia* described here is *E. canis*.

For the neutrophils, we observed a slight increase in the positive by ELISA cases, but there was no statistical difference in any positive or negative group of patients; however, it is important to note that we did not find *Ehrlichia* or *Anaplasma* morulae within any neutrophil, which reduces the possibility of infection by *E. ewingii* or *A. phagocitophylum*, which mostly infects this cellular line (da Silva *et al*., 2016; Quorollo *et al*., 2019). Another important feature we observed was toxic changes in some neutrophils, such as occasional Döhle bodies sometimes with focal basophilia, both findings related to the presence of infectious agents and chronic inflammation (Núñez & Bouda, 2007; Harvey, 2011). Gofton *et al*. (2018) reported the presence of Döhle bodies in positive and negative *Ehrlichia*-like bacteria from platypus blood; however, further studies are necessary.

The total solids use to be increased in *Ehrlichia* infections (Nimsuphan *et al*., 2020), however, we found that the mean value in negative cases barely increased, while in positive cases, the total solids were in range, although a statistically difference was present. In contrast, in the co-infected patients, we found an increase that was significantly different from the *Ehrlichia* positive group, which was expected and comparable to that reported by Saeng-Chuto *et al*. (2016).

It is important to note that, out of all positive cases for *Ehrlichia* spp., 59.92% (157/262) were identified with morulae, indicating that the patients were experiencing acute disease. Our results show that proportionally, there are more animals in the acute phase, which makes them prone to rapidly disseminating the disease to more vectors and animals in contact with them. This is in accordance with the findings of Mylonakis *et al*. (2003) in their study of dogs with clinical signs of acute disease that were positive for the disease. They demonstrated that using the buffy coat in blood smears had a sensitivity of 66% (33/50) for detecting morulae. Therefore, it has been shown that searching for morulae is useful in cases of acute and subclinical courses, as we could detect the disease within the first two weeks of infection, in contrast to ELISA tests that require a certain amount of antibodies in the blood, which are typically achieved around day 24 (Gaunt *et al*., 2010). Another alternative is the use of PCR, which may not be available or cost effective.

Regarding co-infection between *Ehrlichia* spp. and *Anaplasma* spp., in 96% of Tijuana cases, there are several reports of this co-infection in different ecosystems. Ceylan *et al*. (2021) report 6.5% co-infection with these two agents in Turkey; Beristain-Ruiz *et al*. (2022) reported a co-infection rate of 9.27 % in Chihuahua; Bedoya *et al*. (2023) found an 11.9% prevalence by analyzing blood samples from dogs in 22 Mexican states; Aragón-López *et al*. (2021) reported a prevalence of 13.04% in Sonora; and Lara *et al*. (2020) reported 19% co-infection in dogs from veterinary clinics in the Lesser Antilles. As we can observe, in all these cases, a much lower percentage than ours is present, therefore, it is very important to identify the tick species present in the city as well as maintain epidemiologic vigilance of these two rickettsial agents, since they alone can produce severe illness (Piratae *et al*., 2016) and this coinfection can result in a bad forecast.

Competing Interests Statement

The authors declare that they have no competing interests.

Ethics statement

All samples were collected using the standard collection method, without harming the dogs. The research was conducted with the approval of the Ethics Committee of the Veterinary Sciences Research Institute from the Autonomous University of Baja California, registered as CEEIP-201-72024-1. The personal data provided by the owners of the pets sampled in this study were handled in accordance with the Federal Law for the Protection of Personal Data in Possession of Private Parties (DOF 05-07-2010).

Author contribution

All the authors listed have significantly contributed to the development and writing of this article.

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SHORT COMMUNICATION

Replication kinetics of novel swine influenza A viruses: an approach to vaccine production

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ABSTRACT. Novel swine Influenza A viruses (IAVs) have been described in South America. The objective of this study was to evaluate the replication kinetics of novel swine IAVs as a first step in vaccine production. Different swine IAV lineages (H1N1, H1N2, and H3N2), infection doses (MOI: 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001), harvest times (every 12 h), and substrates (MDCK and Vero cells) were used. For all IAV strains, MDCK cells were the most efficient substrate, generating titers of ≥128 HAU/50 µL with an MOI of 0.00001 at 60 h post-infection. These data may be useful in vaccine-producing laboratories.

Keywords: Influenza A virus, MDCK cells, Vero cells, antigen, vaccine, swine.

INTRODUCTION

Novel reassortant H1N2 and H3N2 swine influenza A virus (IAV) strains have recently been identified in commercial farms in Chile. These IAV strains are genetically and antigenically divergent from other IAVs described worldwide and circulate endemically in Chilean swine farms (Tapia *et al*., 2018, 2020). The hemagglutinin (HA) segments of these Chilean IAVs were likely introduced into swine from humans in the late 1980s and the early 1990s (Nelson *et al*., 2015). Other South American countries also have human-origin IAV lineages that circulate endemically in swine (Cappuccio *et al*., 2011; Resende *et al*., 2017). Commercial vaccines based on North American or European IAV strains would not be effective against these IAV strains, reinforcing the need to develop swine IAV vaccines with strains that represent antigenic clusters circulating at the local geographical level (Tapia *et al*., 2020). However, some laboratories do not have the expertise to produce swine IAV vaccines efficiently.

Vaccination is the primary method for preventing and controlling influenza in pigs. Most commercial swine IAV vaccines are based on whole-inactivated viruses, in which the major antigen is HA, a surface glycoprotein (Anderson *et al*., 2016). These vaccines are produced in embryonated chicken eggs or in cell cultures. Embryonated chicken eggs have been widely used in the production of IAV vaccines worldwide for over 70 years (CDC, 2022); however, this substrate has several drawbacks. Dependence on egg supply is a cause for concern, especially during high demand (McLean *et al*., 2016). This requires the availability of a large number of specific-pathogen-free eggs simultaneously and sometimes within a short period of time, which can be scarce or unavailable in some countries. In addition, in resource-limited conditions, using embryonated eggs is labor intensive and requires considerable planning and effort to obtain sufficient eggs for inoculation (Hegde, 2015). Moreover, some IAV strains do not grow well in embryonated chicken eggs, such as human-origin H3N2 strains (CDC, 2019). Importantly, during growth and adaptation to embryonated chicken eggs, through serial passages, IAV strains are likely to acquire mutations that might change their antigenic properties, including glycosylation patterns, which could have an impact on antigenicity and decrease the efficacy of the vaccines produced (Skowronski *et al*., 2014; Zost *et al*., 2017).

Therefore, different mammalian cell lines have been evaluated for the production of whole-inactivated IAV vaccines in the last decade. The cell-based production of influenza vaccines has several advantages. Cell lines can be extensively stored for future production, avoiding dependence on egg supply. The process is more standardized and controlled; therefore, scalability is better with cell culture than with egg-based production systems. In general, mammalian influenza viruses grow well in mammalian cell lines, avoiding the time required for passage and adapting the viruses to embryonated chicken eggs. This also results in a decreased risk of generating mutations during viral passage and allows the maintenance of the antigenic characteristics of these IAVs in cell cultures as compared to embryonated chicken eggs (Manini *et al*., 2017; CDC, 2019; Tree *et al*., 2001). The main mammalian cell lines evaluated for the production of IAV vaccines were Madin-Darby Canine Kidney (MDCK), Af-

rican green monkey kidney (Vero), Per.C6®, and AGE1.CR® (Feng *et al*., 2011; Manini *et al*., 2017; CDC, 2019). Of these, MDCK and Vero cells have been well-studied and licensed for influenza vaccine production (Donis *et al*., 2014; Genzel *et al*., 2010).

The objective of this study was to evaluate the replication kinetics of novel swine IAVs in MDCK and Vero cells and to determine the optimal cell line, initial infectious dose, and harvest time to obtain high antigen (HA) titers. Using this approach, we aimed to establish an efficient protocol for obtaining stable antigen-specific IAV seed strains for use as vaccines against these viruses. In addition to an endemic pandemic H1N1 2009-like (A(H1N1)pdm09-like) swine IAV strain, we used novel reassortant H1N2 and H3N2 swine IAV strains previously identified in commercial farms in Chile.

MATERIALS AND METHODS

We used strains A/swine/Chile/VN1401-274/2014(H1N2), A/swine/Chile/VN1401-4/2014(H1N2), A/swine/Valparaiso/VN1401-559/2014(H1N1), and A/swine/Maule/VN1401-1824/2015(H3N2). They have been previously sequenced and deposited in GenBank (MF099149.1, MF099073.1, MK160103.1, MF099352.1) and genetically characterized. The strains represent four different Chilean swine IAV lineages, as previously described (Tapia *et al*., 2018, 2020). According to the H1 classification described by Anderson *et al.* (2016), the strains A/swine/Chile/VN1401-274/2014(H1N2) and A/swine/Chile/VN1401-4/2014(H1N2) were classified within the clade Other-Human-1B.2, whereas the strain A/swine/Valparaiso/VN1401-559/2014(H1N1) is an A(H1N1)pdm09-like strain classified within clade 1A.3.3.2. The strains A/swine/Chile/VN1401-274/2014 (H1N2), A/swine/Chile/VN1401-4/2014 (H1N2), and A/Maule/Chile/VN1401-1824/2015(H3N2) were genetically distant from other IAVs identified in swine and humans globally (Tapia *et al*., 2018, 2020).

All procedures were approved by the Biosafety Institutional Committee (Certificate Number 104-07-11-2017) and the Institutional Committee for Animal Care and Use (CICUA) of the University of Chile (Certificate Number 02-2016). The study was conducted in accordance with local legislation and institutional requirements. Viral isolation and propagation of viruses were performed under BSL-2 conditions according to international recommendations (Meechan & Potts, 2020).

First, these strains were titrated using a plaque assay, which is an accurate method for the direct quantification of infectious virions by counting discrete plaques (infectious units and cellular dead zones) in cell culture (Baer & Kehn-Hall, 2014). For this assay, 6-well plates were seeded with 600,000 MDCK cells per well, using 3 mL of minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution, and incubated for 24 h at 37 °C and 5% CO_2. The following day, confluent MDCK cells were washed twice with 1x phosphate-buffered saline (PBS) and inoculated with 200 µL of ten-fold serial dilutions of each IAV strain (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10-6). The plates were incubated for 1 h at 37 °C and 5% CO_2 to allow for virus absorption. Then, the inoculum was removed, 2 mL of solid IAV growth medium (MEM, 1% dextran, 5% $NaHCO_3$, 0.3% bovine serum albumin, 2% purified Oxoid™ agar (Basingstoke, Hampshire, UK), and 1 µg/mL trypsin treated with N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)) was added to each well, and the plates were incubated for 48 h at 37 °C and 5% CO_2. Next, 1 mL of 4% formaldehyde was added to each well, and the plates were incubated for 1 h at RT. The solid IAV growth medium with formaldehyde was removed and 0.5% crystal violet was added to visualize the viral plaques in the MDCK monolayers. The plaques were counted, and the virus titer was expressed in plaque-forming units per mL (PFU/mL). Each IAV strain was titrated in triplicates.

Once the titer of each swine IAV strain was determined, 6-well plates were seeded with MDCK and Vero cells (600,000 cells per well) using 3 mL of MEM supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution, and incubated for 24 h at 37 °C and 5% CO_2. The MDCK and Vero cell lines were kindly provided by Dr. Sagar Goyal (University of Minnesota, MN, USA). Confluent cells were washed twice with PBS and inoculated with 200 µL of ten-fold serial dilutions of each strain, corresponding to a multiplicity of infection (MOI) of 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 (MOI refers to the number of virions added per cell during infection, i.e., a MOI of 1 means 1 virion for every cell, while a MOI of 0.00001 means 1 virion for every 100,000 cells). After incubation for 1 h at 37 °C and 5% CO_2, the monolayers were washed twice with PBS, and 3 mL of IAV growth medium (MEM supplemented with 1 µg/mL TPCK-treated trypsin, 0.3% bovine serum albumin, and 1% antibiotic–antimycotic solution) was added. The plates were incubated at 37 °C and 5% CO_2. The monolayers were observed for cytopathic effect (CPE), and 50 µL of supernatant was collected every 12 h post-infection to determine the HA titer using a standard hemagglutination assay (Kitikoon *et al*., 2014). The HA titer was expressed as hemagglutination units per 50 µL (HAU/50 µL) and measured until the CPE exceeded 75% of the monolayer. The most efficient type of cell line, MOI, and harvest time were used to reproduce this assay on a larger scale, using roller bottles with an area of 1,700 cm2 (Corning®, NY, USA). The assay was performed in triplicate.

The MOI of 0.00001 generated the highest HA titer for all IAV strains inoculated in MDCK cells, reaching a peak of 128 HAU/50 µL at 48 h post-infection for the strains A/swine/Chile/VN1401-274/2014(H1N2), and 256 HAU/50 µL at 60 h post-infection for the strains A/swine/Chile/VN1401-4/2014(H1N2), A/Valparaiso/Chile/VN1401-559/2014(H1N1), and A/swine/Maule/VN1401-1824/2015(H3N2) (Figure 1).

In Vero cells, the experiment was extended up to 120 h post-infection due to the slow development of the CPE, at which time the strains reached the maximum HA titer. The strain A/swine/Chile/VN1401-274/2014(H1N2) reached a

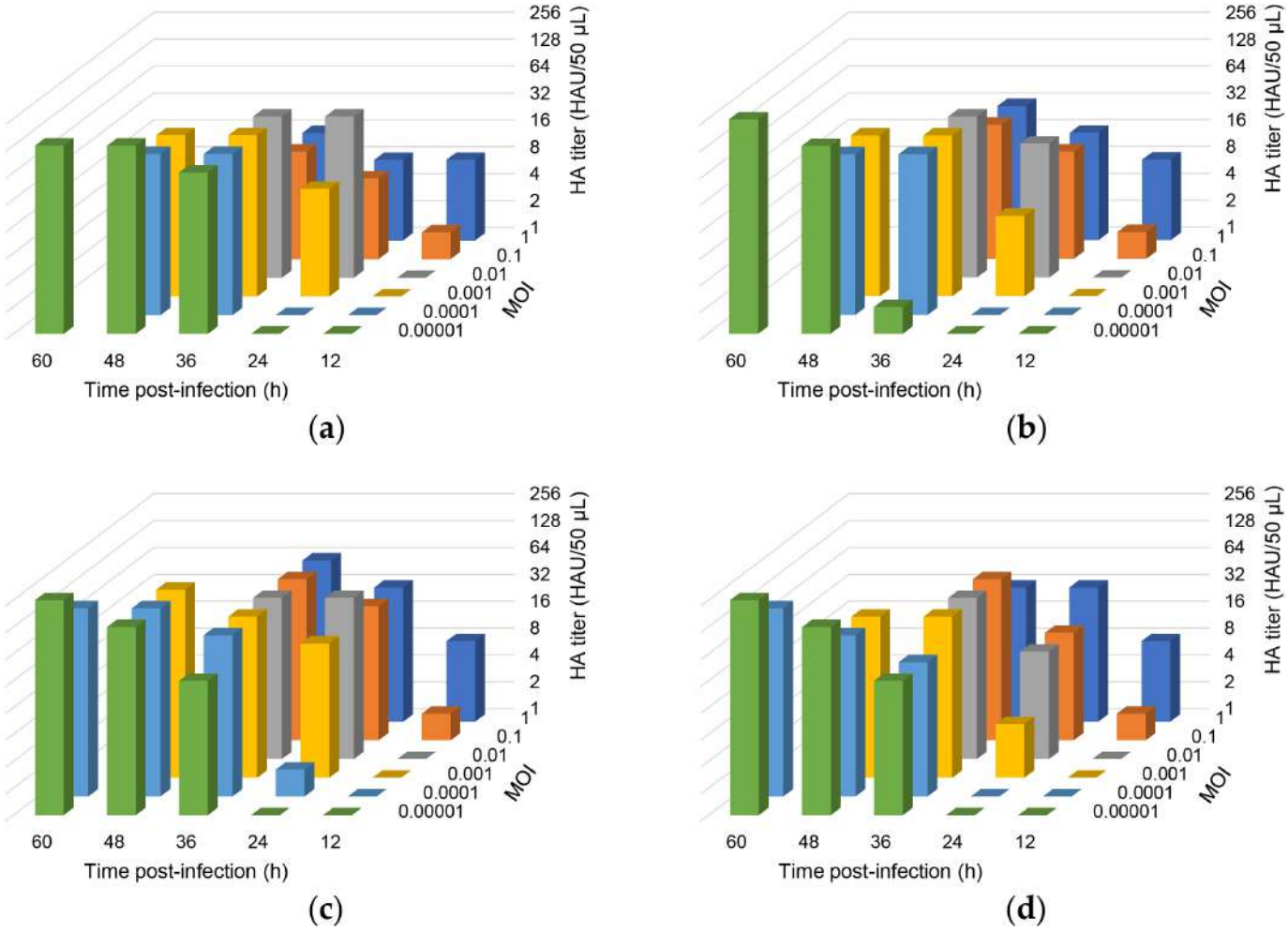

Figure 1.
Replication of swine IAV strains in MDCK cells. MDCK cells were inoculated with strains A/swine/Chile/VN1401-274/2014(H1N2) (a), A/swine/Chile/VN1401-4/2014(H1N2) (b), A/swine/Valparaiso/VN1401-559/2014(H1N1) (c), and A/swine/Maule/VN1401-1824/2015(H3N2) (d). MOI of 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001, were used for each strain. HA titers were calculated at 12, 24, 36, 48, and 60 h post-infection.

titer of 32 HAU/50 µL with the MOI 0.01 and 0.001, whereas the strains A/swine/Chile/VN1401-4/2014(H1N2) and A/swine/Maule/VN1401-1824/2015(H3N2) reached a titer of 16 and 64 HAU/50 µL, respectively, with the MOI 0.1. Strain A/Valparaiso/Chile/VN1401-559/2014(H1N1) did not generate CPE or HA titers (Figure 2).

RESULTS AND DISCUSSION

The MDCK cell line and the MOI of 0.00001 were the most efficient in replicating IAV strains. This infectious dose was then selected to reproduce the assay on a larger scale in roller bottles to confirm the results obtained in the plates. Harvesting was carried out 60 h post-infection. A harvest volume of 300 mL per bottle was obtained for each strain, with a titer of 256 HAU/50 µL for all IAV strains.

In this study, we evaluated the replication kinetics of novel reassortant swine IAV strains and determined the optimal cell line, initial infectious dose, and harvest time for obtaining high HA titers. These strains belong to different clades and subtypes, representing the genetic and antigenic diversity of the swine IAVs circulating in Chile (Tapia *et al*., 2018, 2020).

All IAV strains replicated efficiently in MDCK cells, generating equivalent infection dynamics and HA titers. High HA titers can be achieved using low MOIs. The highest HA titers were obtained at the lowest initial infectious dose (MOI = 0.00001). This inverse relationship between the MOI and virus yield, determined by the HA assay, has been previously described (Isken *et al*., 2012; Petiot *et al*., 2018; Rimmelzwaan

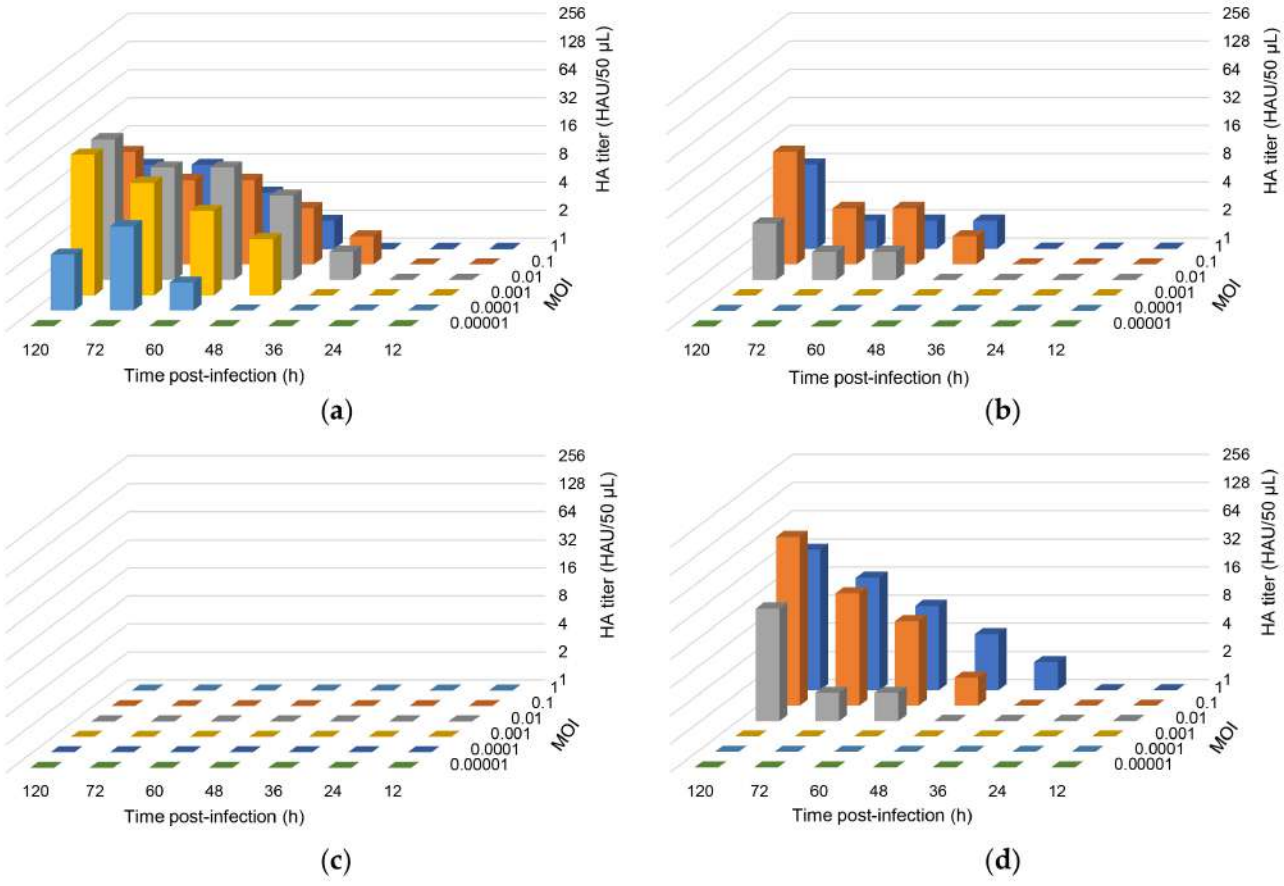

Figure 2.
Replication of swine IAV strains in Vero cells. Vero cells were inoculated with strains A/swine/Chile/VN1401-274/2014(H1N2) (a), A/swine/Chile/VN1401-4/2014(H1N2) (b), A/swine/Valparaiso/VN1401-559/2014(H1N1) (c), or A/swine/Maule/VN1401-1824/2015(H3N2) (d). MOI of 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001, were used for each strain. HA titers were calculated at 12, 24, 36, 48, 60, 72, and 120 h post-infection.

et al., 1998). Low HA titers with high MOIs could be due to the presence of non-infectious biologically active influenza virus particles (niBAPs) or other types of interfering particles that lack replication capacity, specifically non-infectious cell-killing particles (niCKPs) (Brooke, 2014). These non-infectious particles have the potential to influence the course of pathogenesis through their capacity to stimulate or suppress antiviral responses, and in the case of niCKPs, to drive cells into apoptosis (Marcus *et al.*, 2009). These swine IAV strains are likely to generate a high proportion of niCKPs that are sufficiently diluted at low MOIs but play an important role in inducing apoptosis at higher MOIs (Isken *et al.*, 2012). Specifically, this could be the case for strain A/swine/Chile/VN1401-274/2014(H1N2), which reached a lower HA titer (1 log base 2) than the other strains. Possibly, higher HA titers (>256 HAU/50 µL) would have been reached if we used lower MOIs. The cells would have remained viable for a longer period (>60 h), allowing a higher HA titer to be achieved. Isken *et al.* (2012) showed that strain-specific differences in HA titers and the induction of apoptosis are less pronounced with a reduction in the MOI, which is beneficial for robustness in vaccine production processes, as process conditions and harvest time can be kept constant regardless of the strain used (Isken *et al.*, 2012).

In general, IAV strains reached the maximum HA titer at 60 h post-infection in MDCK cells at an MOI of 0.00001. Therefore, this was the optimal harvest time for this MOI. Knowing the optimal harvest time for a given MOI is very useful in vaccine production, as the production of each batch can be better planned and scheduled. It must be noted that, on a large scale, it is not possible to visualize CPE to determine harvest time.

In contrast to MDCK cells, lower HA titers were obtained in Vero cells, indicating less efficient replication kinetics. No CPE or HA titers were obtained with the MOI of 0.00001 at any time post-infection. In fact, the pandemic-like strain A/Valparaiso/Chile/VN1401-559/2014(H1N1) could not replicate in Vero cells. Some IAV strains have been reported

to be unable to generate high viral load titers in Vero cells (Liu *et al*., 2009). This may be because Vero cells rapidly inactivate exogenous trypsin, which restricts the replication of influenza viruses (Kaverin & Webster, 1995). Trypsin has a negative effect on interferon (IFN)-induced antiviral proteins (Seitz *et al*., 2012) and cleaves HA to induce cellular infection (Klenk *et al*., 1975). In addition, canine IFN-induced myxovirus resistance protein 1 (Mx1) produced by MDCK cells cannot inhibit IAV replication (Seitz *et al*., 2010), making it advantageous over other cell lines used in the replication of this virus (Hegde, 2015; Manini *et al*., 2017).

In conclusion, we evaluated the optimal parameters for growth of novel swine IAV strains recently described in Chile. The optimal cell line, initial infectious dose, and harvest time were determined. These factors are critical for vaccine production, particularly for whole-inactivated vaccines. Although the optimal MOI depends on the virus strain, in general, this protocol could be useful for laboratories producing swine IAV vaccines that are just beginning or have less experience. This is important because other South American countries also have human-origin swine IAVs that are genetically and antigenically different from commercial vaccine strains (Cappuccio *et al*., 2011; Resende *et al*., 2017) and might have to produce their own vaccines for effective prevention and control.

DECLARATIONS

Competing interests statement

The authors declare that they have no conflicts of interest.

Ethics statement

Not applicable.

Availability of data and materials

The dataset supporting the conclusions of this study is available in the Supplementary Data.

Authors' contributions

Conceptualization, RT and VN; methodology, RT and RAM; validation, RT and VN; formal analysis, RT; investigation, RT; resources, RT, RAM and VN; data curation, RT; writing—original draft preparation, RT; writing—review and editing, RT, RAM and VN; visualization, RT; supervision, RT and VN; project administration, RT; funding acquisition, RT, RAM, and VN. All authors have read the final version of the manuscript and accept responsibility for the veracity and originality of this work.

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SHORT COMMUNICATION

Use of Delphi methodology to select sustainability indicators on dairy farms: an exploration of environmental, economic, social and animal welfare dimensions

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ABSTRACT. Sustainable livestock production is essential to ensure the availability of food and resources, and to address the social, economic, and environmental challenges that threaten conventional livestock production. While there is consensus among economic, social, and scientific groups on the need to assess sustainability to make decisions that protect resources for present and future generations, there are few sustainability assessment tools that address it holistically. The aim of this study was to develop an assessment tool applicable to farms by identifying the indicators currently applied in dairy farms, based on a systematic literature review and expert opinion. This study used the Delphi methodology to explore sustainability indicators at the farm level. A panel of seven expert researchers and academics in livestock sustainability and animal welfare participated in the study. A high level of consensus was found for 15 economic indicators, 14 social indicators, 20 environmental indicators, and 16 animal welfare indicators. Some indicators, such as financial autonomy, transmissibility, cow and labor productivity, husbandry system, labor intensity, community bonding, labor satisfaction, biodiversity, crop rotation, fertilization, manure management, and water management, showed a high level of consensus and were considered useful in assessing sustainability on dairy farms. In addition, livestock sustainability experts reached a high consensus on 16 animal welfare indicators that could be useful in assessing farm sustainability. These results provide a solid basis for sustainability indicators in the economic, social, environmental, and animal welfare dimensions, which could serve as a basis for developing a sustainability assessment tool for dairy farms.

Keywords: sustainable livestock production; assessment indicators; sustainability dimensions; animal welfare; Delphi methodology.

INTRODUCTION

Continuous access to food and resources is vital for sustaining and advancing all human endeavors. However, livestock farming encounters social, economic, and environmental challenges that jeopardize its capacity to fulfill the current and future requirements of humanity (Munyaneza, 2018). Addressing these challenges necessitates the creation of innovative agricultural technologies and methods that are environmentally sustainable, readily accessible, and efficient for producers, while simultaneously fostering increased food productivity and mitigating adverse impacts on human and animal health (Velten *et al*., 2015; Henning & Jordaan, 2016).

Given the variety of impacts that conventional livestock production can have on social, economic, and environmental aspects, sustainable livestock production has gained relevance in recent years (Zahm *et al*., 2008). Although significant advances in crop and livestock productivity have been made it is still uncertain if it will be possible to sustain this progress in the future (Velten *et al*., 2015)

The sustainability challenges encountered in livestock farming may exhibit variation across different countries, regional contexts, or even production systems (Castillo-Rodríguez *et al*., 2012; Salinas, 2014). For example, in High income countries, key challenges in terms of sustainability include diversification of a limited range of commodities and addressing environmental concerns raised by social groups, especially about significant nutrient loss and excessive use of pesticides (Zhen & Routray, 2003). Conversely, in countries with lower and middle incomes, the primary aim is to sustain food production while conserving the current resource foundation (Munyaneza, 2018).

In every sustainability initiative, it's essential to have a clear understanding of the operational definition of the "sustainability concept" to initiate any project (Munyaneza, 2018; Zahm *et al*., 2008). Even if the fundamental essence of sustainability is apparent, its practical implementation can differ based on various individual viewpoints (Munyaneza, 2018; Seghezzo, 2009). The word "sustainable" originates from the Latin word "subtenir," which translates to "to sustain" or "to support from below" (Munyaneza, 2018). The most recognized definition of sustainability or sustainable development offered by the World Commission on Environment and Development (WCED, 1987), is the "development that meets the needs of the present without compromising the ability of future generations to meet their

own needs" (chap. II, para. 1). However, some authors have pointed out that this definition is subjective creating a problem when trying to apply it (Munyaneza, 2018; Brunett *et al*., 2006; Van Passel *et al*., 2007; Ruiz *et al*., 2017). On the other hand, a more recent definition has been proposed by Broom (2014) who defines sustainability as: "A system or procedure is sustainable if it is acceptable now and if its expected future effects are acceptable, in particular in relation to resource availability, consequences of functioning, and morality of action", emphasizing other aspects of sustainability such as the morality of action, and considering a system as not sustainable if it, for example, has negatives effects on animal welfare (p. 353).

An essential difficulty arises in delineating the concept of "need," as what one person regards as essential requirements, others might view as mere desires (Munyaneza, 2018; Cox & Ziv, 2005). This ambiguity suggests that what may be deemed sustainable for one individual could be deemed moderately or entirely unsustainable for another (Munyaneza, 2018). Furthermore, Seghezzo (2009) highlights additional shortcomings in the definition of sustainability, including its anthropocentric perspective, the excessive emphasis on economic factors, and the neglect of environmental, social, and animal welfare dimensions.

Sustainability assessment through indicators is proposed as the approach to implement the concept of sustainability (Munyaneza, 2018; Van Passel *et al*., 2007; Binder *et al*., 2010; Broom, 2021). Presently, multiple methodologies have been created to evaluate the sustainability of cattle production on individual farms (Attia *et al*., 2021; Salinas, 2014; Meul *et al*., 2012; Verduna *et al*., 2020; Pérez Lombardini *et al*., 2021), however, these tools may not be suitable for the farm reality of different countries. One solution to this problem is to adapt existing methodologies to the specific context of the country in which they are applied (Munyaneza, 2018; Ruiz *et al*., 2017).

The selection of indicators is considered a critical step during sustainability assessment, as these have an impact on the conclusions and outcomes of interventions. Factors affecting sustainability outcomes can be diverse, such as household characteristics, different types of farms, and different environments where livestock farming takes place (Munyaneza, 2018; Van Calker *et al*., 2008). Therefore, understanding these factors is critical to guide any intervention aimed at improving sustainability. For the selection of sustainability indicators, various methodologies have been utilized, including expert-driven and stakeholder-driven approaches (Munyaneza, 2018; Gómez-Ravelo *et al*., 2013).

One methodology that can be used to obtain a consensus of informed opinions from subject matter experts, overcoming individual limitations and reflecting a complete and broad view of sustainability, is the Delphi method (Gómez-Ravelo *et al*., 2013). The Delphi method involves assembling a panel of experts within the pertinent research field, rather than employing a random sample representative of a target population (Ahmad & Yew Wong, 2019; Keeney *et al*., 2001). In contrast to household surveys, there is not a standard sample size requirement for the Delphi technique (Munyaneza, 2018; Henning & Jordaan, 2016). Regarding the number of experts involved, Varela-Ruiz *et al*. (2012) propose that a range of seven to 30 experts is necessary to ensure reliable outcomes.

This paper applies the Delphi methodology, a reliable top-down approach to reach consensus and unify criteria on a specific topic, to select the most appropriate indicators to create a sustainability assessment tool for dairy cattle farms.

MATERIAL AND METHODS

The study was conducted between January and April 2023. For the selection of indicators, an initial list was compiled via a systematic review of scientific literature, which included indicators currently used to assess livestock sustainability in economic, social, environmental, and animal welfare dimensions.

This initial list, used as the basis for the Delphi survey, consisted of 24 indicators for the economic dimension, 19 indicators for the social dimension, 31 indicators for the environmental dimension, and 16 indicators for animal welfare.

Afterward, the survey was distributed via email to 20 researchers. These specialists were chosen due to their qualifications, background in the dairy industry, proficiency in sustainable livestock agriculture, accessibility, and eagerness to engage in the survey.

The Delphi survey consisted of one round in which the list of indicators was initially presented to the experts. Each indicator was evaluated in terms of usefulness, ease of evaluation, cost of implementation, and importance in sustainability. The experts scored the indicators using a 3-point scale (1= Not useful at all; 2= Useful; and 3= Very useful). Once the questionnaires from the first round were collected, mean scores, standard deviation, and coefficient of variation were calculated for each indicator (Figure 1).

The degree of agreement achieved plays a crucial role in determining whether additional rounds are necessary in the research process (Henning & Jordaan, 2016; Zinn *et al*., 2001). To gauge the levels of consensus, the coefficient of variation (CV) was utilized, following the classifications outlined by English and Kernan (1976) and Henning and Jordaan (2016), where we considered: a good level of consensus, indicating no need for further rounds ($0 \leq CV \leq 0.5$); a less satisfactory consensus, possibly warranting another round ($0.5 < CV \leq 0.8$); and a poor level of consensus, necessitating another round ($0.8 \leq CV$).

Furthermore, the standard deviation serves as a metric for evaluating the spread of values within a population (Grobbelaar, 2007). In this research, the degree of agreement identified by Grobbelaar (2007) and subsequently by Henning and Jordaan (2016) was utilized as a reference for making decisions based on the standard deviation (SD), with classifications as follows: high consensus ($0 \leq SD \leq 1$); reasonable or acceptable consensus ($1.01 \leq SD \leq 1.49$); low consensus ($1.5 \leq SD \leq 2$); and no consensus ($2 \leq SD$).

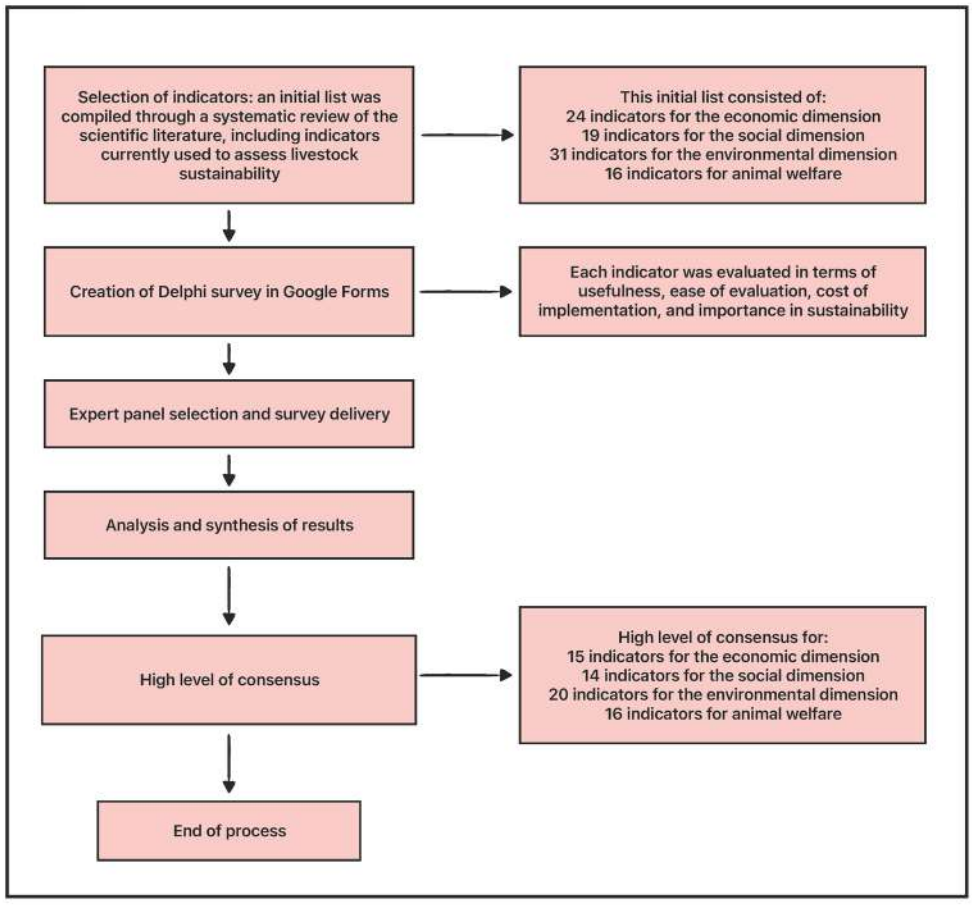

Figure 1.
Flowchart of Delphi methodology used in the study.

RESULTS AND DISCUSSION

Concerning the number of experts, a response rate of 35% was obtained, which corresponds to seven experts. According to Varela-Ruiz *et al*. (2012), the minimum number of experts to obtain valid results in the Delphi methodology is seven, so despite this response rate, it was possible to apply the methodology in the study. In order to achieve an adequate selection of livestock sustainability indicators, the expert panel was composed of researchers in livestock sustainability and animal welfare, dairy industry actors familiar with the components and challenges of livestock sustainability from Chile, Colombia, Brazil, Uruguay and Argentina, together with one person from Holland.

Once the experts' initial responses were obtained, the coefficient of variation (CV) rule proposed by English and Kernan (1976) was applied to measure the level of consensus and the need for a second round. It was observed that all indicators presented a high and satisfactory level of consensus ($0 \leq CV \leq 0.8$) for each of the dimensions. This means that it was not necessary to resort to a second round among the experts (Table 1).

Next, the standard deviation (SD) analysis, proposed by Grobbelaar (2007), was carried out for each indicator to determine which of them would be part of the final farm sustainability assessment tool. This analysis resulted in a high level of consensus for 15 economic indicators, 14 social indicators (Table 1), 20 environmental indicators, and 16 animal welfare indicators (Table 1).

Henning and Jordaan (2016) similarly utilized the standard deviation approach to ascertain consensus within the Delphi methodology. They aimed to identify the criteria utilized by banks in providing credit to farmers and producers. Like the current study, their findings suggested that employing standard deviation (SD) yielded reliable outcomes in assessing the level of consensus among Delphi methodology experts.

Table 1 displays the indicators that achieved a high level of consensus among the experts. Notably, for economic sustainability, accounting indicators are prominent, along with indicators aiding in the assessment of production costs, consistent with the findings of Henning and Jordaan

(2016). Moreover, Galioto *et al*. (2017) highlighted that the predominant economic indicators in dairy cattle farms revolve around the correlation between production costs and equivalent liters of milk, as well as profitability indicators encompassing intangible aspects like product quality and production methods. These conclusions align with the results of the current study and are corroborated by prior research conducted by Munyaneza (2018), Galioto *et al*. (2017),

Table 1.
Sustainability indicators for the economic and social dimensions were obtained by applying the Delphi methodology. The mean ($\bar{x}$), standard deviation (SD) and coefficient of variation (CV) of the scores for each indicator are provided.

	Economic dimension	$\bar{x}$	**SD**	**CV**
1	Economic viability	2.86	0.58	0.20
2	Financial autonomy	2.00	0.58	0.29
3	Transmissibility	1.86	1.15	0.62
4	Efficiency of production processes	2.00	0.58	0.29
5	Income per liter of milk	2.86	0.58	0.20
6	Cow productivity	3.00	0.00	0.00
7	Labour productivity	3.00	0.00	0.00
8	Feed conservation	2.57	1.00	0.39
9	Animal disease control (vaccination and parasite control)	2.86	0.58	0.20
10	Breeding system	2.71	1.15	0.43
11	Forage self-sufficiency	2.29	1.15	0.51
12	Safety, quality and transparency of production activities	2.43	0.58	0.24
13	Gross dairy farm income	2.71	1.15	0.43
14	PIB contribution	1.86	1.15	0.62
15	Land productivity	2.57	1.00	0.39
	Economic specialization rate *	2.00	1.53	0.76
	Sensitivity to government aid *	1.43	1.53	1.07
	Income over feed cost *	2.14	2.65	1.23
	Herd vigilance *	2.00	1.53	0.76
	Profitability *	2.29	1.53	0.67
	Feed cost expenditure *	2.43	1.53	0.63
	SOCIAL DIMENSION	$\bar{x}$	**SD**	**CV**
1	Level of schooling	3.00	0.00	0.00
2	Work intensity	2.71	0.58	0.21
3	Quality of life	2.86	0.58	0.20
4	Community involvement	2.71	0.58	0.21
5	Collective work	2.43	0.58	0.24
6	Hygiene and safety	2.57	1.00	0.39
7	Level of training of the farm manager	2.57	0.00	0.00
8	Generational transition	2.71	0.58	0.21
9	Risk of abandonment	2.57	1.00	0.39
10	Work satisfaction	2.71	0.58	0.21
11	Economic dependence	2.14	0.00	0.00
12	Diversification of activities on the farm	2.71	0.58	0.21
13	Labor rights	2.86	0.58	0.20
14	Cultural diversity	2.43	0.58	0.24
	Employment generation *	2.43	1.53	0.63
	Quality of facilities *	2.29	1.53	0.67
	Empowerment of women *	2.43	1.53	0.63
	Labor efficiency *	2.29	1.53	0.67

*Indicators that reached a low level of consensus in the Delphi methodology are shown in italics. These indicators were eliminated from the final sustainability list.

Table 1. (Continued)
Sustainability indicators for the environmental and animal welfare dimensions were obtained by applying the Delphi methodology. The mean ($\bar{x}$), standard deviation (SD) and coefficient of variation (CV) of the scores for each indicator are provided.

	Economic dimension	$\bar{x}$	**SD**	**CV**
1	Crop biodiversity	2.29	0.58	0.25
2	Animal biodiversity	2.43	1.15	0.48
3	Crop rotation	2.43	1.15	0.48
4	Grassland área	2.71	0.58	0.21
5	Organic waste management	2.43	1.15	0.48
6	Space valorization	2.14	1.00	0.47
7	Fertilization	2.71	0.58	0.21
8	Manure, slurry and wastewater management residuals	2.71	0.58	0.21
9	Pesticide use	3.00	0.00	0.00
10	Soil resource protection	3.00	0.00	0.00
11	Water resource management	3.00	0.00	0.00
12	Energy efficiency	2.86	0.58	0.20
13	Water quality management	2.57	0.00	0.00
14	Phosphorus balance	2.14	1.00	0.47
15	Specialization	1.71	1.00	0.58
16	Greenhouse gas production	2.57	1.00	0.39
17	Habitat consevation	2.71	0.58	0.21
18	Disposal of milk from animals that received medication	2.43	0.58	0.24
19	Air Quality	2.29	0.58	0.25
20	Carbon sequestration per kg of milk	2.57	1.00	0.39
	Global warming potential *	2.14	2.00	0.93
	Acidification *	2.00	1.53	0.76
	Eutrophication *	2.14	2.00	0.93
	Nitrogen balance *	2.43	1.53	0.63
	Energy balance *	2.43	1.53	0.63
	Animal welfare	$\bar{x}$	**SD**	**CV**
1	Body condition	3.00	0.00	0.00
2	Access to wáter	3.00	0.00	0.00
3	Water quality and water trough cleanliness	2.71	0.58	0.21
4	Dimension of milking stalls and milking parlors	2.86	0.58	0.20
5	Floor condition	3.00	0.00	0.00
6	Cleanliness score of udders	3.00	0.00	0.00
7	Cleanliness score of hindquarters	3.00	0.00	0.00
8	Condition of holding pen floor	3.00	0.00	0.00
9	Presence of shade	3.00	0.00	0.00
10	Teat condition score	3.00	0.00	0.00
11	Absence of cow with tail injuries	2.86	0.58	0.20
12	Locomotion score	3.00	0.00	0.00
13	Use of analgesics and anesthetics in painful procedures	3.00	0.00	0.00
14	Pain management in acute illness	3.00	0.00	0.00
15	Expression of positive social behaviors	3.00	0.00	0.00
16	Flight zone distance	2.71	0.58	0.21

*Indicators that reached a low level of consensus in the Delphi methodology are shown in italics. These indicators were eliminated from the final sustainability list.

Zucali *et al.* (2016), Salinas (2014), and da Silva and Gameiro (2022).

For environmental sustainability, consensus was notably achieved for 20 indicators, including crop rotation, manure management, land fertilization, and energy efficiency (Table 1). Previous studies have highlighted a significant concern regarding the greenhouse gas emissions associated with conventional livestock farming practices, prompting a shift towards mitigating these environmental impacts to foster more sustainable production. Mitigation strategies outlined in literature include minimizing or eliminating tillage practices in favor of alternative land preparation methods for specific crops (Smith *et al*., 2001; Bacenetti *et al*., 2015), implementing crop rotation (Cederberg *et al*., 2005), adopting targeted fertilization techniques (Smith *et al*., 2007; Eckard *et al*., 2010), selecting appropriate seed varieties (Evans, 1996), and converting cropland to pasture (Soussana *et al*., 2009). These indicators also garnered high consensus levels in the current study.

Regarding the social dimension, a substantial consensus was observed for 14 indicators, addressing not only the welfare of workers but also their job satisfaction levels and the potential risk of turnover (Table 1). While the social dimension holds significant importance in sustainability assessments, it tends to be the least defined and often overlooked aspect. Numerous authors emphasize that evaluating social aspects ensures the continuity of livestock farming across generations (da Silva & Gameiro, 2022; Verduna *et al*., 2020; Broom, 2021; Pérez-Lombardini *et al*., 2021).

The 16 indicators proposed for evaluating animal welfare through the Delphi method have garnered significant consensus among the participating experts, as outlined in Table 1. This achievement can be partly attributed to the consensus among various authors regarding the importance of animal welfare in assessing sustainability in livestock farming (Broom, 2021; da Silva & Gameiro, 2022; Pérez-Lombardini *et al*., 2021; Zucali *et al*., 2016) and the growing significance of animal care in consumers' decisions regarding animal-derived products (Miller *et al*., 2020). The attention directed towards this issue stems from the shared understanding that enhancing animal welfare contributes to optimal health, ultimately leading to increased profitability in dairy production (Galioto *et al*., 2017; Broom, 2021; Arvidsson Segerkvist *et al*., 2020).

Finally, it is crucial to highlight the limited number of studies dedicated to the identification and development of sustainability metrics. Most of the research related to sustainability has evaluated pre-existing methodologies, and the results obtained have varied depending on the methodology used and the areas where they were applied.

Although the number of participants in the methodology was low, and some participants may be more familiar with some particular dimensions of sustainability, the Delphi method did allow to identify pertinent indicators to assess the sustainability of dairy farms across housed and pasture systems. These indicators can be analysed by generating an index for each dimension, in order to balance the differences in the number of indicators included in each of them (environmental, economic, social and animal welfare) and make them comparable. It is expected that this assessment will help to identify both strengths and areas for improvement, thereby improving dairy farming practices in economic, social, environmental, and animal welfare terms.

In conclusion, the use of a top-down approach, as the Delphi methodology, does allow to identify sustainability indicators with a good level of consensus. This set of indicators can be later on used by producers or researchers to assess the sustainability of dairy farms. Feedback from experts is essential for developing reliable assessment tools in many areas, and their participation in this type of methodologies is crucial.

DECLARATION

Declaration of interests

The authors declare that there is no conflict of interest.

Author contributions

MSH: conceptualization, research, analysis, and writing. TT: writing, revising, and editing. All authors contributed to the writing and discussion of the manuscript and approved its final version.

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CASE REPORT

Cranial osteomyelitis associated with *Pasteurella canis* in broiler chickens

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ABSTRACT. Species of the *Pasteurella* genus are part of the oropharyngeal microbiota of many animal species. In poultry, *Pasteurella multocida* causes fowl cholera, the chronic manifestation of which can include neurological symptoms. *Pasteurella canis* causes osteomyelitis and other infections in humans. To the best of our knowledge, this is the first report of cranial osteomyelitis associated with *Pasteurella canis* in broiler chickens in Chile.

Keywords: poultry, infections, zoonotic pathogen, dogs, *Pasteurella Canis*

Cranial osteomyelitis, otitis, swollen head syndrome, and meningoencephalitis in poultry are associated with various infectious agents, including *Avibacterium paragallinarum* (Crispo *et al*., 2018), *Ornithobacterium rhinotracheale* (Banani *et al*., 2015; Al-Hasan *et al*., 2021), and particularly *Pasteurella multocida* (Boulianne *et al*., 2020). Species of the *Pasteurella* genus are part of the normal oropharyngeal microbiota of many animal species and cause multiple infectious diseases in a wide range of organisms, including humans and poultry (Wilson & Ho, 2013). *Pasteurella multocida* and *P. canis* are the main *Pasteurella* genus species associated with human diseases following septic bite wounds and inflammation at the injury site (Gautier *et al*., 2005). *Pasteurella spp*., which belongs to the *Pasteurellaceae* family, are small, non-motile, facultative anaerobic, gram-negative coccobacilli measuring 1–2 µm in length (Boulianne *et al*., 2020). *Pasteurella multocida* is the causative agent of fowl cholera, a contagious disease affecting domestic and wild birds, and mortality usually ranges from 0% to 20% in naturally infected chickens (Boulianne *et al*., 2020). The main clinical signs of acute presentation are fever, anorexia, ruffled feathers, mucoid discharge from the mouth, diarrhea, and an increased respiratory rate. Chronic presentation can cause torticollis and opisthotonos due to meningeal infection (Boulianne *et al*., 2020). *Pasteurella canis* is part of the normal microbiota of healthy companion animals, particularly dogs. On several occasions, *P. canis* infections have been reported in humans and are associated with osteomyelitis and cutaneous abscess in the right digit (Hara *et al*., 2002), soft tissue infection (Kim *et al*., 2016), breast implant infection (Hannouille *et al*., 2019), abdominal infection (Mensah-Glanowska *et al*., 2020), septic arthritis of the femorotibial joint (Nascimento *et al*., 2021), and, recently, endophthalmitis (Bathula *et al*., 2023). *Pasteurella canis* has also been associated with endocarditis in dogs (Kern *et al*., 2019) and pneumonia in black-tailed marmosets (Da Silva *et al*., 2020).

To our knowledge, this is the first report of cranial osteomyelitis associated with *P. canis* infection in broiler chickens in Chile, and it is supported by *pre-* and *post-mortem* findings, bacterial culture, biochemical characteristics, and histopathological analysis.

Between September 2022 and August 2023, the Avian Pathology Laboratory at Universidad de Chile received 35 broiler chickens aged 34–45-d-old for diagnostic evaluation following the sudden onset of neurological symptoms. The fowl came from a commercial broiler farm in the Libertador General Bernardo O'Higgins Region, where the condition affected 4% of the birds, showing a slight increase in mortality, which was quickly controlled through treatment with amoxicillin administered in drinking water at 20 mg/kg body weight daily at 12 h intervals for 5 days. All broiler chickens received live attenuated vaccines against infectious bursal disease (IBD) and avian infectious bronchitis (IB) at one day of age.

Prior to necropsy, the chickens presented severe disorientation (n = 31), torticollis (n = 29), opisthotonos (n = 29), and difficulty standing (n = 21) (Figure 1). They were euthanized by cervical dislocation, and the gross post-mortem examination revealed mild nasal discharge (n=32), caseous material, and fragility in the cranial bone (n = 29), mainly in the ventral area close to the ear (Figure 2). No other significant injuries were observed in the outer, middle, or inner ear; upper respiratory system; joints; or internal organs.

Samples of the brain, cranial bone, nasal discharge, and middle ear of the affected birds were obtained aseptically, plated on tryptone soy agar containing 5% blood and

Figure 1.
Broiler chicken with severe disorientation, displaying symptoms of torticollis and opisthotonos, and difficulty standing.

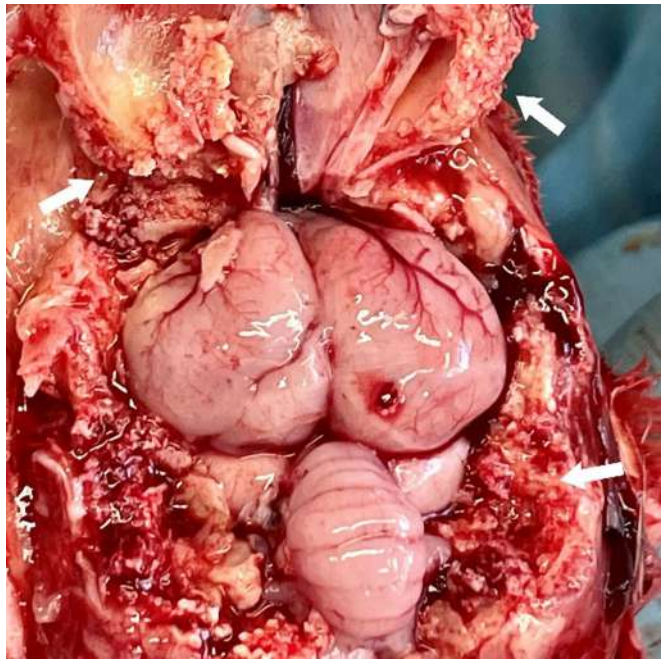

Figure 2.
Macroscopic lesions in affected birds. Cranial osteomyelitis with the presence of caseous material (white arrows).

MacConkey agar, and incubated in 5% CO_2 at 37°C for 24 h. Cranial bone (occipital) sections (n = 5) were collected, decalcified with 3% nitric acid, fixed in 10% neutral-buffered formalin for three days, embedded in paraffin wax, cut into 4 µm sections, stained with hematoxylin and eosin, and examined under a light microscope.

Brain samples were aseptically collected and homogenized in phosphate-buffered saline (PBS; pH 7.2) 10% containing 200 U/mL penicillin and 0.2 mg/mL streptomycin. Homogenates were vortexed for 10 s, subjected to three freeze-thaw cycles, and centrifuged at 3000 × *g* for 20 min at 4 °C. The supernatant was transferred to a sterile tube and preserved at –80 °C to rule out the presence of Newcastle disease virus (NDV) and avian influenza virus (AIV) infection by viral isolation and hemagglutination assays (Alexander, 2000). Nasal discharge samples were collected to exclude the presence of *Mycoplasma synoviae* and *Mycoplasma gallisepticum* by polymerase chain reaction (PCR) under previously reported conditions (Lauerman, 1998).

After 24 h incubation on blood agar plates, cultures from the cranial bone (n = 29), brain (n = 20), and middle ear (n = 10) presented smooth, grayish-white, mucoid, non-hemolytic colonies compatible with *Pasteurella spp*. (Figure 3). No bacterial growth was observed on MacConkey agar plates. No colonies compatible with *Pasteurella* were observed in nasal discharge samples. Pure subcultures were obtained from single colonies on a blood agar plate for Gram staining, catalase and oxidase tests, and biochemical testing using the Vitek® 2 Compact identification system (BioMerieux, Marcy-l'Étoile, France) according to the manufacturer's instructions. The latter identified the species as *Pasteurella canis* with an accuracy of 99 %. The main results of the biochemical testing that identified *Pasteurella canis* isolated in this study, distinguishing it from other species of the *Pasteurella* genus, such as *Pasteurella multocida*, *Pasteurella stomatis*, and *Pasteurella dagmatis* (Christensen & Bisgaard, 2024), are detailed in Table 1. All the isolates were gram-negative.

Histopathological examination of cranial bone samples revealed a chronic inflammatory response and dense infiltration of inflammatory cells, including heterophils that form granular eosinophilic aggregates surrounded by macrophages, along with extensive bone resorption and necrotic bone tissue. Several bacteria were observed in the center of the lesions. These microscopic alterations were consistent with granulomatous osteomyelitis (Figure 4). The NDV and AIV testing by viral isolation and hemagglutination assay were negative, as were PCR analyses for *Mycoplasma synoviae* and *Mycoplasma gallisepticum*. Given the macroscopic and microscopic lesions observed, characteristics of the isolated colonies, Gram staining, and biochemical testing of the isolates obtained from brain, cranial bone, and middle ear samples, we confirmed the diagnosis of cranial osteomyelitis associated with *Pasteurella canis*. Antimicrobial susceptibility testing was performed using the disk diffusion method (Kirby-Bauer) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines to determine the antimicrobial susceptibility of *Pasteurella canis* isolated from the cranial bone (Table 2).

Table 1.
Results of the main biochemical tests on the *Pasteurella canis* strains isolated in this study and other species of the *Pasteurella genus*.

Test	*Pasteurella canis*	*Pasteurella multocida*	*Pasteurella stomatis*	*Pasteurella dagmatis*
Catalase	+	+	+	+
Oxidase	+	+	+	+
Indole production	+	+	+	+
Urease	-	-	-	+
Ornithine decarboxylase	+	+	-	-
Lysine decarboxylase	-	-	-	-
Acid from:				
D-mannitol	-	+	-	-
Dulcitol	-	V	-	-
D-sorbitol	-	V	-	-
Maltose	-	-	-	+
D-arabinose	-	V	-	V

+: Positive; -: Negative; V: Variable

Neurological symptoms such as severe disorientation, torticollis, opisthotonos, and difficulty standing could cause significant losses for the poultry industry owing to poor feed conversion ratios, higher mortality, and increased slaughterhouse condemnation of broilers. Like any infectious disease, it causes immunological stress that drives down food consumption, keeps affected birds from feeding properly, delays growth, and lowers the weight of chickens in slaughterhouses (Boulianne *et al*., 2020).

Figure 3.
On 5% blood agar, after 24 h incubation, smooth, grayish-white colonies were observed.

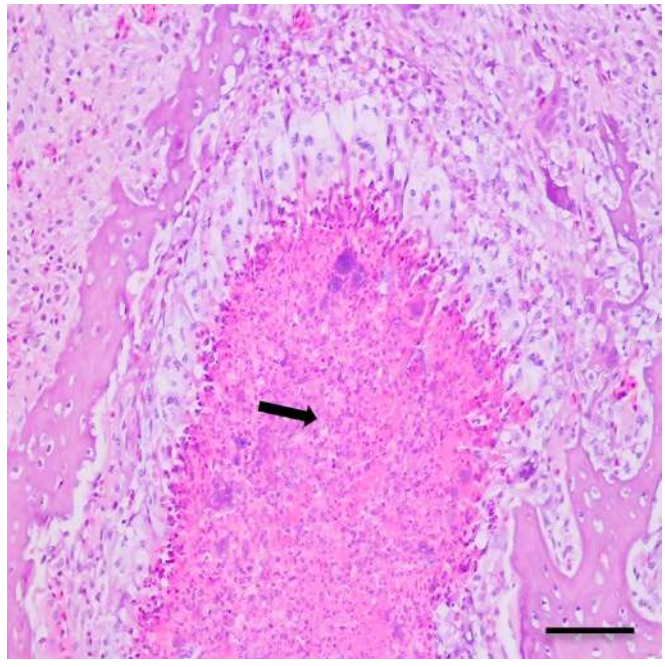

Figure 4.
Histological lesions of affected broiler chickens. Granulomatous osteomyelitis is observed with the presence of inflammatory cells. There is extensive bone resorption and necrotic bone tissue. Several bacteria are observed in the center of the lesion (black arrow). H&E. Bar = 100 µm.

Table 2.
Antimicrobial susceptibility test results for *Pasteurella canis* isolate from cranial bone.

Antimicrobial	Inhibition halo (mm)	Interpretation
Amoxycillin	31	Sensitive
Colistin	15	Sensitive
Enrofloxacin	31	Sensitive
Streptomycin	10	Resistant
Florfenicol	33	Sensitive
Fosfomycin	30	Sensitive
Fosfomycin + Tylosin	25	Sensitive
Lincomycin	0	Resistant
Lincomycin-Spectinomycin	20	Sensitive
Norfloxacin	29	Sensitive
Oxytetracycline	23	Sensitive
Sulfadoxine-Trimethoprim	28	Sensitive
Sulfisomidine	0	Resistant
Tiamulin	15	Intermediate
Tylosin	16	Intermediate

Pasteurella canis, formerly known as *P. multocida* biotype 6, or 'dog type' (Mutters *et al.*, 1985), is part of the normal microbiota of the oral cavity of dogs and cats (Wilson & Ho, 2013). Two biotypes of *P. canis* have been described: biotype 1 is mainly observed in the oral cavities of dogs, whereas biotype 2 is isolated from calves. Biotype 1 is also normally isolated from bite wounds of carrier animals (Gautier *et al.*, 2005). Dogs and cats found near farms are likely sources of infectious agents in poultry, such as *Pasteurella canis*.

Although middle and inner ear infections are uncommon in birds (Shivaprasad *et al.*, 2006), pathogens can migrate from the nasal turbinate, oral cavity, or infraorbital sinus, and extend into the middle ear through the ear canal to colonize the inner ear. Another possibility is that *Pasteurella canis* spreads from the sinuses to the adjacent air-filled skull bones, with subsequent necrosis and the onset of neurological symptoms, producing secondary ear infections (Boulianne *et al.*, 2020). This could explain the absence of any evident inflammation in the inner, middle, or outer ear despite the isolation of *Pasteurella canis* in the middle ear samples. Although the isolated *Pasteurella canis* strain is susceptible to 10 of the 15 antimicrobials tested, its presence in animals intended for human consumption could constitute a serious public health issue. Given its ability to cause infection without a bite or direct inoculation, it can potentially become a zoonotic foodborne pathogen (Hannouille *et al.*, 2019). Furthermore, the use of antimicrobials to control infection increases the risk of antimicrobial resistance owing to the selective pressure of antibiotics and their limited availability for poultry farming. All isolated *Pasteurella canis* strains were sensitive to amoxicillin, consistent with decreased mortality after treatment.

As pathogens can be transmitted directly or indirectly via different routes, the design and implementation of biosecurity measures must consider all possible pathogens and production chain entry routes. In poultry production, biosecurity measures focus primarily on preventing highly transmissible exotic diseases (e.g., avian influenza and Newcastle disease) and foodborne zoonotic diseases caused by bacteria that are part of the normal intestinal microbiota of birds (e.g., *Salmonella* and *Campylobacter*) (Souillard *et al.*, 2024). However, biosecurity measures must also control pathogenic agents with uncommon entry routes, particularly those transmitted by biological vectors, such as dogs and cats living near or inside poultry farming facilities. These vectors include workers' companion animals, wild cats, and guard dogs, which carry pathogens with great zoonotic potential that could emerge through poultry farming, such as *Pasteurella canis*.

DECLARATIONS

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Competing interests

The authors declare that they have no competing interests.

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