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Cryptosporidium spp. detection in water tanks and ponds of dairy farms

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ABSTRACT

Objective: To identify *Cryptosporidium* spp. in water tanks and ponds of dairy farms from the Comarca Lagunera.

Design/Methodology/Approach: The experiment was carried out in dairy farms from the Comarca Lagunera, a region that extends on both sides of the border between the states of Coahuila and Durango, Mexico. Water samples from 36 open-air ponds (with or without polyethylene covers) and four stainless-steel tanks were analyzed using a modified version of the conventional Ziehl-Neelsen method. In addition, n=28 feces samples were taken from 10 dairy farms.

Results: The modified Ziehl-Neelsen method was used to identify *Cryptosporidium* spp. in five water samples (12.5%) out of the 33 samples taken from open-air ponds without a polyethylene cover. *Cryptosporidium* spp. was not detected in the 28 remaining water samples (84.9%). In addition, the parasite was not detected either in the three water samples from open-air ponds with a polyethylene cover or in the four samples from the stainless-steel tanks. Meanwhile, *Cryptosporidium* spp. was found in 57% of the 28 feces samples.

Limitations/Implications: Molecular studies should be carried out to identify the *Cryptosporidium* species found.

Findings/Conclusions: The parasite in question was detected in some open-air ponds of the dairy farms under study using the conventional diagnosis method. Future research should include an analysis to determine if the species found are zoonotic, in order to prevent potential diseases among humans and animals.

Keywords: Zoonoses, Protozoan, Cattle, Mexico.

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INTRODUCTION

Cattle production has a worldwide impact: millions of families work in dairy production. In addition, it provides food security to countries and households and it is a major source of income (FAO, 2023). The domestication of cattle took place about 10,000 years ago, providing humankind with a survival and development alternative. Furthermore, the fast development of technology has transformed dairy production into one of the main food industries (Silanikove *et al.*, 2015).

The five countries with the highest dairy production are India, USA, China, Pakistan, and Brazil (FAO, 2023). For its part, Mexico holds the eighth place among worldwide producers. Meanwhile, the Mexican states of Jalisco, Coahuila, Durango, and Chihuahua

account for 50% of the total domestic production (SIAP, 2023). In northern Mexico, parts of Coahuila and Durango make up an area called Comarca Lagunera. This region is one of the main producers in the country. The arid climate and scarce precipitation of the dairy basin result in a high production. In addition, the intensive production systems of the area are highly technified —from automatic milking systems to milking handling preserving cold chain conditions—, enabling their growth and development (Gallegos-Daniel *et al.*, 2023).

From the epidemiological point of view, zoonotic diseases have a major impact on domestic animals. These diseases can be transmitted to humans directly or through fomites and they can be transmitted or propagated through water (Thompson *et al.*, 2016). Bacteria, viruses, and protozoa are three etiological agents that cause waterborne diseases. Protozoa are the less regulated agent, because conventional water treatments do not tackle their cystic form. *Cryptosporidium* spp. is included in this group. This parasite causes cryptosporidiosis, an emergent disease identified by the American authorities in 1993, after an outbreak that took hundreds of human lives in Milwaukee, Wisconsin (Doménech, 2013).

Studies about water in the Comarca Lagunera region are scarce and, consequently, no information about water pollution related to *Cryptosporidium* spp. is available. Therefore, this study proposed that water ponds and tanks that supply water to calves have *Cryptosporidium* spp. oocysts. Consequently, identifying the cryptosporidia in water is fundamental to determine if it is a source of transmission in dairy herds.

MATERIALS AND METHODS

The study was carried out in September 2021 and May 2023 in the Comarca Lagunera. This region is located in both sides of the border between the states of Coahuila and Durango (25° 33' 00" N and 10° 26' 00" W).

Water samples from open-air ponds of 40 regional dairy farms were analyzed. The samples were taken from September to November 2021. Each 4 L sample was taken from the opposite side of the water inlet. A layer of organic matter was identified at the pond's edge. It was carefully removed to take a water sample from the area. Previously disinfected plastic containers were used for the sampling. These containers were placed at ≈4 °C in a cooler and taken to the lab. The water sample information included if they were taken from a pond covered with oilcloth or polyethylene covers or if the tank had a concrete bottom. Water samples from farms without a water pond were collected from stainless-steel tanks.

Water sample analysis

The samples were allowed to settle for 24 h at room temperature in the Unidad de Diagnóstico Veterinario lab of the Universidad Autónoma Agraria Antonio Narro - Unidad Laguna. Afterwards, a pump was used to suck the supernatant and then it was sieved with a .0017 inches (#325) test sieve. Approximately 80 ml of sediment were finally recovered. Subsequently, the recovered sediment was centrifuged at 2,500 xg for 10 minutes, in 10 ml tubes with screw tops. The supernatant was sucked and the sediment

was suspended in a 2.5% potassium dichromate solution (5:1 ratio). Afterwards, the water samples were centrifuged again at 2,500 xg for 10 minutes. A 2 ml aliquot was taken from each sample and was placed in 2.5 ml microcentrifuge tubes for their analysis. The solution rested for 1 h. Subsequently, 50 μL were taken from the bottom of the tubes and were placed in a microscope slide. The samples were air dried and stained with the modified Ziehl-Neelsen method (mZN) (García *et al.*, 1983). A Coplin staining jar was used to filter carbol fuchsin, before the slides were treated for 30 minutes. Afterwards, they were washed with running water and immersed in acid-alcohol-resistant for 10 seconds. Subsequently, they were washed with running water and immersed in methylene blue for five minutes. Finally, they were again washed with running water and air-dried. Once they were dried, the slides were treated with xylol and placed in coverslips. Light microscopy with a 40x magnifying glass was used to visualize the samples, comparing the shape, size, and redness of oocysts of a positive control previously identified in a cow feces sample.

Collection and analysis of feces samples

In addition to the water samples collected in the area, feces samples were taken from 28 calves (1-20 days old). The handing of the calves used in this complied with the technical specifications for the production, care, and use of animals in the lab (SAGARPA, 2001). A bottle labelled with the name of the farm, earing cattle number, and date of birth of the calves was used to collect the feces sample. The samples were kept in a cooler (2-8 °C).

Two grams of the feces from each sample were placed on a microscope slide. The material was used to carry out an extension. Subsequently, the samples were air-dried and stained with the modified Ziehl-Neelsen method (mZN) (García *et al.*, 1983). A Coplin staining jar was used to filter carbol fuchsin, before the slides were treated for 30 minutes. Afterwards, they were washed with running water and immersed in acid-alcohol-resistant for 10 seconds. Subsequently, they were washed with running water and immersed in methylene blue for five minutes. Finally, they were again washed with running water and air-dried. Once they were dried, the slides were treated with xylol and placed in coverslips. Light microscopy with a 40x magnifying glass was used to visualize the samples, comparing the shape, size, and redness of oocysts of a positive control previously identified in a cow feces sample.

Data analysis

Descriptive statistics were used to analyze the positive and negative values of samples obtained from water ponds, water tanks, and feces that recorded a positive result with the modified Ziehl-Neelsen method. Inferential statistics were used to establish the relationship between water storage conditions (open or closed water tank or pond, with or without a polyethylene cover, or with a concrete bottom) and the positive and negative results of the feces samples subjected to a water analysis. The Chi-Square test (χ^2) was used to calculate the ratio between the categorical variables, using the InfoStat-Statistical Software (2020). The significance level was $P<0.05$.

RESULTS AND DISCUSSION

Cryptosporidium spp. was found in five samples (12%) in tanks without covers. These samples were part of the 36 samples taken from water ponds with and without polyethylene covers (Table 1). Meanwhile, no oocysts were found in four samples taken from water tanks.

A total of 28 feces samples were taken from 10 dairy farms. *Cryptosporidium* spp. was found in 16 samples (57%) from nine dairy farms: seven samples (25%) from five dairy farms were positive for *Cryptosporidium* spp. in water; and nine samples (32%) from four dairy farms, were negative in water. In addition, the remaining 12 samples tested negative for *Cryptosporidium* spp. (Table 2).

Number of samples examined (n=28) Number of positive samples

Using the staining method of the mZN, this study found *Cryptosporidium* spp. in the open-air ponds of five dairy farms (12.5%). No oocysts were found in dairy farms with closed water storage systems, such as stainless-steel tanks. These closed systems are less likely to be polluted by *Cryptosporidium* spp. oocysts from the environment, because they are protected from winds, wildlife (such as waterfowl and landbirds), and insects that could carry oocysts to dairy farm water sources (Åberg *et al.*, 2020). According to Wu *et al.* (2020), *Cryptosporidium* spp. is a single-cell protozoan parasite that causes gastroenteritis to humans and animals. For their part, Zhou *et al.* (2003) pointed out that water is a reservoir for *Cryptosporidium* spp. and other microorganisms.

The 36 water samples subjected to the mZN were divided into two groups: samples from water ponds with polyethylene covers and samples from water tanks with a concrete bottom and without polyethylene covers. The plastic infrastructure of the tanks of three

Table 1. *Cryptosporidium* spp. found in water samples. Five out of 40 samples recorded a positive diagnosis using the modified Ziehl-Neelsen method. They accounted for 12.5% of the total. Significance: P<0.05.

Water	Samples examined (n=40)	Positive samples	p-value
Tank	4	0	
Pond	36	5 (12.5%)	0.4396
Pond with cover	3	0	0.4824
Pond without cover	33	5 (15.1%)	

Table 2. *Cryptosporidium* spp. found in feces samples. Sixteen out of 28 samples recorded a positive diagnosis using the modified Ziehl-Neelsen method. They accounted for 57% of the total. Significance: P<0.05.

Feces	Samples examined (n=28)	samples positive	p-value
Five stables were positive for *Cryptosporidium* spp. in the water	14	7 (25%)	0.4450
Five stables were negative for *Cryptosporidium* spp. in the water	14	9 (32%)	

dairy farms recorded negative results. Eighty-six percent of the negative samples were from dairy farms with tanks without coating. These results are the consequence of the periodical preventive management of the pond, the biosecurity measures followed to prevent diseases in the farms, and the disease treatment protocols applied to the animals (Cleere *et al.*, 2017).

Meanwhile, the mZN was used for a presumptive cryptosporidiosis diagnosis of neonatal animals. The results indicated a total prevalence of 57%. Out of this total, 32% of the feces samples from dairy farms tested positive to *Cryptosporidium* spp., although, according to the mZN, the protozoa were not found in water. In the case of these dairy farms where the parasite was found in the feces but not in their water sources, the animals could have been infected by direct fecal-oral transmission, bad husbandry practices, or housing animals of different ages in the same stables (Agrawal *et al.*, 2023). Nevertheless, 25% of the positive feces samples came from dairy farms where, according to the mZN, *Cryptosporidium* spp. was present in their water sources. Therefore, the animals were probably infected by water from polluted ponds; however, water is not the only source of transmission of oocysts and the subsequent infection (Ikiroma and Pollock, 2021).

Cryptosporidium spp. are commonly found in calves at an early age. This study was carried out in ten different dairy farms, with ±20 days old animals (Diaz *et al.*, 2004). Although the samples were not taken in the same day, this study confirmed the presence of the causative agent of cryptosporidiosis (Qi *et al.*, 2020).

CONCLUSIONS

Cryptosporidium spp. has a worldwide distribution and impacts a large number of species. Using a modified version of the Ziehl-Neelsen method, this study detected its presence in water samples from ponds and tanks where calves drink. Future research should identify the *Cryptosporidium* species found in the Comarca Lagunera to determine if they are zoonotic or not. This protozoan causes major economic losses and pollutes groundwater, consequently infecting the human population. In addition, dairy farms should implement a plan to prevent this infection.

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