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Presence of *Mycobacterium avium* subspecies *paratuberculosis*, in goat's milk, natural rennet and fresh cheese from San Luis Potosi, Mexico

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***Mycobacterium avium* subspecies *paratuberculosis* (Map) is the etiological agent of *paratuberculosis*, which is one of the chronic diseases that cause financial losses in livestock production. Map can be present in cheese and other dairy products, especially those made with unpasteurized milk. Contamination of the food supply chain exposes humans to the bacteria, making the disease an important zoonosis of public health significance and a one-health emergency. The purpose of the study was to determine the presence of Map in raw goat milk, natural rennet, and artisanal fresh cheese. A total of 18 milk samples were collected directly from the bulking tank, 23 from fresh cheese, and 10 from milk rennet from five municipalities in the State of San Luis Potosi, Mexico. Samples were analyzed through bacteriological culture and IS900 PCR-n. Statistical analysis was carried out in STATA® 7.0, analyzing frequencies and the Kappa test to determine the concordance index between bacteriological culture and IS900 PCR-n results. Map was isolated from four milk samples (n=4/18, 22%), one from cheese (n=1/23, 4.3%), while none were obtained from rennet samples. IS900 PCR-n detected 22 positive samples: 6/18 (33.3%) in milk, 8/10 (80%) in rennet and 8/23 (34.74%) in cheese. Concordance between IS900 PCR-n and bacteriological culture in milk samples was high (0.7273) but low in cheese samples (0.0707). Map was detected in milk and artisanal cheese, although it is noteworthy that Map genomic material was detected in 80% of rennet samples analyzed with PCR. Quality control of milk, rennet, and all the inputs used for making cheese is necessary.**

Key words: Paratuberculosis, map, fresh milk, cheese, rennet, goats

INTRODUCTION

Handcrafted dairy products, traditionally and due to their characteristics related to their presentation, have great acceptance among the Mexican population. Handcrafted cheese production in Mexico is varied; approximately 40

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varieties of cheese are known, with the production of fresh-type cheese standing out (Sánchez-Valdés et al., 2016). Because these products are made without standardized methods, technification is low and facilities tend to be inadequate, thereis a greater risk for using milk and rennet that are contaminated with pathogens such as *Mycobacterium avium* subspecies *paratuberculosis* (Map), which is the etiological agent of paratuberculosis and is considered one of the chronic diseases that cause financial losses in livestock production. The disease is characterized by causing granulomatous enteritis, and an elimination pathway mostly through stool, that contaminates water, and feed, as well as through colostrum and milk making it the main infection and transmission pathway in newborns and suckling kids (Idris et al., 2022; Cirone et al., 2006).

Goat's milk, beyond being an important part of lactating kids, is also used to make cheese for human consumption. In various rural regions in the Mexican Republic, it is common to find artisanal cheese, in other words, made with unpasteurized milk and using non-sterile rennet, this is obtained from the abomasum of nursing calves or kids, cut into fragments, sun dried, placed in plastic containers with whey and left to ferment for five days for later use. This practice can be an important source of contamination with pathogens (Rodríguez-Gallegos et al., 2022).

Several studies have reported that Map can be present in cheese and other dairy products, especially those that have been made with unpasteurized milk, although its viability depends on several factors such as maturation time, pH, and salt concentration therefore viability ranges between 8 to 27 weeks (Hosseiniporgham et al., 2022; Feitosa de Albuquerque et al., 2017; Hanifian 2014; Cirone et al., 2006). Confirmation of paratuberculosis is carried out with bacteriological culture from tissues with lesions, stool, milk, and cheese. A large variety of solid and liquid culture media have been used for Map, which is a slow-growing microorganism that requires between 6 to 20 weeks to develop colonies (Whittington et al., 2013; Grant et al., 2006).

Currently, there are molecular techniques such as PCR (Polymerase Chain Reaction) to identify DNA from Map in samples obtained from milk, mesenteric lymph nodes, intestine, and fecal matter of suspected and clinically sick animals. The most widespread primers used for PCR are those that amplify a fragment of the 900 Insertion Sequence (IS900) that is specific to Map (Jaimes et al., 2008; Cirone et al., 2006).

Deficient sanitary management of dairy goat herds, as well as inadequate processes in making cheese, carry an increasing risk of contamination with microorganisms that depreciate the product and put human health at risk (Feitosa de Albuquerque et al., 2017). The purpose of the study was to determine the presence of Map in samples from goat's milk, artisanal rennet and cheese.

MATERIALS AND METHODS

Non-random, convenience sampling was carried out from products of 23 cooperative producers from the following five municipalities in the State of San Luis Potosi, Mexico: Villa Juarez (n=1), La Ventana (n=5), Cerritos (n=10), Villa Arista (n=6), and Guadalcazar (n=1). A total of 18 samples were collected from bulk milk tanks using 50 ml Falcon plastic tubes, 23 from recently made fresh-type cheese in plastic bags, and 10 from rennet, also in Falcon tubes. A questionnaire was used to determine the characteristics of the production units.

Bacteriological culture

Five ml were taken from each sample of milk and rennet; while for the cheese, 2 g were macerated using 5 ml sterile water and placed in 50 ml polypropylene tubes. All samples were decontaminated using the acid-alkali method and inoculated in duplicate in Herrold's Egg Yolk agar with added mycobactin (2 mg/L) and amphotericin B (20 mg/L). Samples were incubated for 16 weeks at 37°C. Bacterial smears were done from any tube with bacterial growth and stained with Ziehl-Nielsen for acid-fast bacilli (Payeur et al., 1993).

pH measurement

Before bacteriological culture was carried out, pH was measured of the milk, curd and cheese samples using an OAKTON pH 500 series pH meter.

DNA extraction and IS900 nested PCR (IS900 PCR-n)

DNA was extracted from milk, rennet and cheese samples following the methods described by Gurrola et al. (2018), while the IS900 PCR-n was carried out following the methods by Jaimes et al. (2008). The initial PCR was done using IS900 primers Paratb1 (5´-TGA TCT GGA CAA TGA CGG TTA CGG A-3´) and Paratb4 (5´-CGC GGC ACG GCT CTT GTT-3´), that yield a 563 base-pair (bp) fragment. The nested PCR was done using primers Paratb2 (5´-GCC GCG CTG CTG GAG TTG A-3´) and Paratb3 (5´-AGC GTC TTT GGC GTC GGT CTT G-3´), that yield a 210 bp fragment. DNA from Map strain ATCC #700535 was used as positive control. Expected amplicons were visualized in 2.0% agarose gels stained with ethidium bromide.

Statistical analysis

Statistical analysis was done using STATA® 7 software package (StataCorp LP, College Station, TX, USA). Analysis of detection frequencies by IS900 PCR-n and bacteriological culture of milk, cheese and rennet samples was carried out. Kappa analysis was used to determine the concordance index between the results of IS900 PCR-n and bacteriological culture.

RESULTS

Characteristics of production units

The production system of the participating units was determined to be mixed type since animals were set out

Table 1. Mean pH values of samples from milk, cheese, and natural rennet from goats.

Sample	Mean pH
Milk	6.5
Fresh cheese	6
Natural rennet	4.9

to graze during the day and brought in to corrals at night where they are milked in the morning. Milking is done on areas with dirt floors without sanitation of the corrals before or after milking, thus fecal matter remained for some time in the milking area.

Milking is done by hand and only teats are cleaned before milking without nipple pre-sealing or end-sealing being done. All producers stored the milk in bulk milk tanks, which were cleaned daily. Cheese was made using unpasteurized milk and natural rennet. Milk was kept at ambient temperature until used, which usually happens a couple of hours after milking. Cheese was kept refrigerated until sold, which was done in the local area. None of the producers carried out quality control bacteriological testing of the milk or rennet.

Health conditions of the herds entailed vaccination only against *Brucella* and annual deworming. Paratuberculosis diagnosis has never been carried out; therefore the prevalence of the disease in the herds was unknown.

pH measurement in milk, fresh cheese, and natural curd

Average pH values from milk, fresh cheese and natural rennet were found to be within the normal range established for inputs used for making fresh-type cheese, slightly acidic (Table 1).

Bacteriological culture of Map from milk, fresh cheese, and natural rennet samples

Four Map isolates were obtained from milk samples (n=4/18, 22.22%) and one from fresh cheese (n-1/23, 4.3%), while none was obtained from natural rennet. Bacterial colony growth was observed from the sixth week of incubation in Herrold's Egg Yolk agar with added mycobactin. Staining affinity and bacterial morphology were confirmed with Ziehl-Neelsen's stain observing acid-fast bacilli (Table 2)

IS900 PCR-n from DNA obtained from milk, fresh cheese, and natural rennet samples

A total of twenty two positive samples were detected: 6/18 (33.3%) from milk, 8/10 (80%) from rennet, and 8/23 (34.78%) from cheese samples (Table 2). Positive samples amplified the 210 bp amplicon that corresponds to the IS900 insertion sequence specific to *Mycobacterium avium* subspecies *paratuberculosis*. Concordance between IS900 PCR-n and bacteriological culture of milk samples was high (0.7273) with a p-value of 0.0007 which was significant, while concordance between both tests with that of cheese samples was low (0.0707). It was not possible to calculate concordance between tests with rennet samples as no Map growth was observed and the number of rennet samples was limited (Table 3).

DISCUSSION

Quality of raw materials (milk and natural rennet) that are used to make artisanal cheese should encompass aspects such as health and sanitary conditions, chemical and physical composition, and organoleptic characteristics of the product. Nevertheless, artisanal cheese production in rural communities does not include these practices due to lack of awareness of good manufacturing practices which risk cheese becoming contaminated with pathogens.

Several studies on sanitary quality of cheese report several contaminant microorganisms that are present in these products and are a risk for human health, such as: *Escherichia coli* O157:H7 and other fecal coliforms, *Clostridium botulinum, C. perfringens, Staphylococcus aureus,* emetic type *Bacillus cereus, Vibrio cholerae, V. parahaemolyticus, Yersinia enterocolitica, Shigella sp., Salmonella spp.,* and *Listeria monocytogenes*, among others. Their presence in cheese depends on the quality and thermal treatment of milk, cleanliness of the cheese factory, quality of cultures, handling of the rennet during processing, and storage temperature (Rodríguez-Gallegos et al., 2022; Sánchez-Valdés et al., 2016). Detection of these microorganisms is important but the detection of Mycobacteria such as *M. bovis* (bovine milk) and Map (bovine, goat, and sheep milk) should be included in the detection panel as well since it is known that bovine tuberculosis is considered a public health issue (Gurrola-Mejía et al., 2018), and in the case of Map, there are studies in the literature and in progress that associate it as part of the etiology of Crohn's disease associated with consumption of milk or cheese with traces of Map DNA is considered a risk factor (Hosseiniporgham et al., 2022; Feitosa de Albuquerque et al., 2017; Hanifian, 2014; Cirone et al., 2006).

Detection of Map in fecal, tissue or milk samples through bacteriological culture and/or PCR indicates that paratuberculosis is present in the herd (Whittington et al., 2013). The results in this work show the presence of Map in goat herds in the study region from which milk and rennet are obtained to make cheese. Artisanal cheese

Table 2. Bacteriological culture and IS900 PCR-n tests of goat´s milk, fresh cheese, and rennet samples from San Luis Potosi, Mexico.

Variable	Map bacteriological culture		IS900 PCR-n	
	+ (%)	-(%)	+ (%)	-(%)
Milk (bulk tank)	4 (22.2)	14 (77.8)	6 (33.3)	12 (66.7)
Natural rennet	0 (0)	10 (100)	8 (80.00)	2 (20.00)
Fresh cheese	1 (4.3)	22 (95.7)	8 (34.78)	15 (65.22)

STATA® 7.0

Table 3. Concordance between IS900 PCR-n and bacteriological tests of goat's milk, cheese, and rennet samples..

Diagnostic test pairs	K	SE	Z	p
IS900 PCR-n and culture, milk samples	0.7273	0.2268	3.21	0.0007
IS900 PCR-n and culture, cheese samples	0.0707	0.1495	0.47	0.3182
IS900 PCR-n and culture, rennet samples	ND*	ND*	ND*	ND*

K, Kappa; SE, Standard error; Z, Z-score; p, p value. STATA® 7.0; *Not determined.

made from raw milk in general is made either from bovine or goat milk, spontaneously fermented, or with short maturation time using non-standardized rudimentary methodologies. Amongst dairy products, fresh cheese has the greatest number of pathogens present at the time of marketing. For this reason, it is most frequently associated with food poisoning outbreaks (Sánchez-Valdés et al., 2016). It is thought that Map survives in milk and cheese due to factors such as pH, time of maturation and salt concentration (Hanifian, 2014; Cirone et al., 2006). In this study, fresh cheese that is sold and eaten in less than two days after manufacturing, was sampled one week after manufacture. The pH of the unpasteurized milk and cheese (6.5 and 6, respectively) make it possible for Map to remain viable. Also, PCR-n testing detected the presence of Map in the rennet, which could be due to the origin of the kids or calves, from which the abomasum was obtained, from herds with paratuberculosis and either were fed with colostrum or milk contaminated with Map, or could have been born already infected (Idris et al., 2022; Cirone y col. 2006). Map was not isolated from rennet, most probably due to decreased viability of Map bacilli at pH 5, which is usually the level found in natural rennet. Also, the sampling was small and there needs to be more than 100 viable bacilli per milliliter to be able to obtain a positive culture (Hanifian, 2014; Cirone et al., 2006). Map has been previously detected in various dairy products and sub-products. One study carried out in Switzerland detected the presence of Map using IS900 PCR in 19.7% (273/1384) of milk samples collected from bulk milk tanks (Corti and Stephan, 2002). Slana et al. (2009) found, in a study done in Cyprus, 63 (28.6%) of 220 samples from bulk milk tanks to be positive to Map using IS900 and F57 real-time PCR. Our results match those of previous studies since 33.3% of milk samples were determined to be positive for Map using IS900 PCR and 22.2% using bacteriological culture. Detection of Map using bacteriological culture is considered the gold standard, although the sensitivity is low (<50%). Map can readily be detected with approximately 10 times more sensitivity using PCR techniques. The apparent mismatch between methods could be explained by low Map concentration in the sample, since PCR is a more sensitive technique and does not require the bacilli to be viable to obtain DNA regardless of the sample used (feces, tissues, milk, cheese) (Feitosa de Albuquerque et al., 2017; Bauman et al., 2018).

The high frequency of samples positive to paratuberculosis could be because milk samples were obtained from herds that have a high paratuberculosis seroprevalence. It is important to note that the real seroprevalence could be higher than estimated because not all animals shed the bacterium through milk (Feitosa de Albuquerque et al., 2017). Bovines, ovine, and caprine with subclinical paratuberculosis infection could shed low amounts of Map through milk making it essential to use adequate diagnostic tests that are sensitive enough to detect the bacterium. The sensitivity of PCR for detecting Map in milk samples had been shown to vary between 1 and 100 CFU/mL of milk (Feitosa de Albuquerque et al., 2017). When samples were taken for this study, the livestock producers did not know if their herds had paratuberculosis since they have never carried out such diagnostic testing. This makes it necessary to carry out an epidemiological study of paratuberculosis in the herds to determine their health status and the risk factors in the region to be able to establish appropriate control

measures.

The presence of paratuberculosis in dairy goat herds is due to various factors such as sharing spaces with other animal species, either domestic (sheep and cattle) or wild animals, that are infected with Map, especially due to the management of fecal matter which can contaminate water and food, and that Map can survive up to 365 days in favorable temperature and humidity conditions. Also, females infected with paratuberculosis can develop mastitis and shed bacilli through milk. If such milk is not pasteurized, Map can remain viable for up to 100 days in bulk milk tanks and 60 days in cheese (Rodríguez-Gallegos et al., 2022; Cirone et al., 2006). A study carried out in Brazil with “Coalho” artisanal cheese detected Map-specific DNA in 10% (3/30) of cheese samples and Map was isolated from 3.3% (1/30) of samples (Faria et al., 2014). Clark et al. (2006), reported in their study carried out in Wisconsin which analyzed 98 cheese samples to detect Map by PCR and bacteriological culture, that it was not possible to isolate Map, but 5% of the samples were positive by PCR.

The presence of Map in cheese depends on the quality and thermal treatment of milk, general sanitation status of the cheese factory, handling of the rennet during processing, and storage temperature.

Conclusion

In this study, Map was detected in samples from milk and artisanal fresh cheese. It is noteworthy that 80% of rennet samples analyzed by PCR testing were found to have Map DNA. This makes it necessary to have good quality control of milk and rennet, as well as all inputs used to make cheese.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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