



SHORT COMMUNICATION

Relationship between Microscopic Agglutination Test and Real-Time PCR Assay for Detection of Seropositivity of Pathogenic *Leptospira* Infections in Cattle in Chile: A Pilot Study

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Abstract

Objectives: This study was aimed at evaluating the relationship between microscopic agglutination test (MAT) seropositivity and real-time polymerase chain reaction (PCR) reactivity in cattle. An additional objective was assessment of the diagnostic value of both tests in detecting seropositivity and infection caused by pathogenic *Leptospira*.

Methods: Blood and urine samples were collected from 61 bovines in the Los Lagos region of Chile. The MAT detected a panel of eight *Leptospira* serogroups, whereas real-time PCR was conducted with a TaqMan probe targeting the *Leptospira* lipL32 gene.

Results: The seropositivity rate for pathogenic *Leptospira* was 39.3% (95% confidence interval [95% CI], 27.04–51.57) according to the MAT, and *Tarassovi* and *Sejroe* were the most frequently detected serogroups. The antibody titers ranged from 1:200 to 1:800. The positivity rate determined with real-time PCR was 29.5% (95% CI, 18.05–40.94), and the leptospiral concentrations ranged from 1.07 to 12,500 leptospires per milliliter urine. Among the 24 animals with MAT-reactive serum samples, 18 also had urine samples with positive real-time PCR results; thus 75% of animals had positive results with both techniques. The kappa coefficient between tests of 0.784 (95% CI, 0.539–1.0) indicated substantial agreement. The sensitivity and specificity of real-time PCR versus MAT were 75.0% (95% CI, 57.7–92.3) and 100%, respectively. The positive and negative predictive values were 100% and 86.7% (95% CI, 75.7–96.4), respectively.

Conclusion: The results demonstrated the complementary nature of MAT and real-time PCR analyses in diagnosing bovine leptospirosis: combined use of both tests enhanced diagnostic accuracy. Real-time PCR detected *Leptospira* infection and bacterial renal excretion, thus providing insights into the presence and extent of environmental contamination, whereas the MAT identified seropositivity, antibody titers, and the serogroups associated with infection.

Key words: cattle, diagnostic tests, kappa coefficient, MAT, pathogenic *Leptospira*, real-time PCR, sensitivity, specificity

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Received: January 14 2025

Revised: March 4 2025

Accepted: March 21 2025

Published Online: April 12 2025

INTRODUCTION

Leptospirosis, a globally distributed zoonotic disease caused by spirochetes of the genus *Leptospira* [1], poses substantial public health concerns due to morbidity and mortality in humans [2]. In cattle, the acute form of the disease can cause fever, hemolytic anemia, hematuria, hemoglobinuria, jaundice, and meningitis [3,4]. Chronic leptospirosis in cattle is associated with reproductive disorders, including infertility, stillbirths, abortions, and the birth of weak offspring [5].

Because a substantial proportion of infected cattle remain asymptomatic, laboratory tests are required to confirm seropositivity or active infection [6]. Diagnostic approaches include general laboratory analyses; indirect methods such as the microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay; and direct detection techniques, including darkfield microscopy of biological samples (e.g., blood, urine, and cerebrospinal fluid), bacterial culture, and molecular methods such as polymerase chain reaction (PCR) [7].

Among serological tests, the MAT is the reference method, because its high specificity enables identification of *Leptospira* serogroups [8]. The test involves mixing a serum sample with serial dilutions of antigens containing strains representing the predominant serogroups in a given geographic region. The serum-antigen mixture is then incubated, observed under darkfield microscopy, and assessed for agglutination. The degree of agglutination indicates the antibody titer, with a reference threshold of 50% agglutination [9]. *Leptospira*-specific serogroup antibodies typically appear 1 week post-infection and can persist for months or years, thus enabling detection via the MAT [10].

A recent systematic review and meta-analysis of human leptospirosis cases in the Americas has identified Icterohaemorrhagiae, Canicola, Pomona, and Grippotyphosa as the most frequently identified serovars [11]. Globally, the predominant *Leptospira* serovars in various animal species include Grippotyphosa, Icterohaemorrhagiae, Pomona, Australis, and Ballum [12]. Epidemiological studies in South America have indicated that Sejroe is among the most common serogroup in cattle, and have reported leptospirosis seroprevalence rates of 39.8% in Brazil [13], 16.0% in Colombia [14], and 19.5% in Argentina [15]. PCR assays can detect specific nucleotide sequences of the leptospiral genome in blood, urine, or tissue samples with high sensitivity. Real-time PCR is particularly advantageous, because it can quantify synthesized DNA through fluorescence measurement [16,17]. Additionally, real-time PCR has lower contamination risk than conventional PCR and therefore is a preferred diagnostic method [16]. Detection of leptospiral nucleic acid in blood or tissues confirms infection, whereas its presence in kidney tissue or urine indicates either infection or colonization [18]. The lipL32 gene, which encodes an outer membrane lipoprotein and is a virulence factor for pathogenic

leptospirae, is a common target in real-time PCR protocols [19,20].

This pilot study was aimed at evaluating the relationship between the MAT serological reaction and real-time PCR positivity in cattle, and assessing the diagnostic utility of both tests in detecting pathogenic *Leptospira* infection and seropositivity.

METHODS

This cross-sectional, quantitative, descriptive observational study [21] was conducted at the Veterinary Clinical Hospital of Universidad San Sebastián, Puerto Montt (41.4683°S, 72.9461°W), and a farm in Los Muermos (41.3945°S, 73.4649°W) in the Los Lagos region of southern Chile. The study included 61 female Holstein and Jersey cattle 2–10 years old, because leptospirosis is particularly relevant in this age range, owing to its association with reproductive complications such as abortions [22]. Cattle were included if they exhibited a clinical and physiological state suitable for sampling; were not vaccinated against *Leptospira*; and showed no clinical signs of the disease. A probabilistic sampling method was used [23]. All cattle treated at the Veterinary Clinical Hospital during 6 months and those from the farm were included in the study ($n = 61$).

Blood samples (1.5 mL) were collected via jugular venipuncture and placed in sterile tubes without anticoagulant. The samples were centrifuged at 3,000 rpm for 5 min to obtain 0.5 mL serum, which was stored at -20°C until analysis. Urine samples were collected after the induction of urination. At least 10 mL urine samples were obtained, stored at 4°C – 6°C , and analyzed within 4 days.

The MAT was performed on each serum sample to detect anti-*Leptospira* antibodies and identify the serogroup responsible for seropositivity. The protocol described by Faine [24] and WHO-ILS [25] was followed, with the panel of pathogenic *Leptospira* species, serogroups, serovars, and reference strains detailed in Table 1. Antibody titers of 1:100, 1:200, 1:400, 1:800, and 1:1,600 were assessed. A titer of 1:100 was classified as seropositive for pathogenic *Leptospira* [25]. If a sample reacted to multiple serovars with identical titers, it was considered to demonstrate co-agglutination [26].

DNA was extracted and purified from each urine sample with a High Pure DNA Kit (Roche) according to the manufacturer's instructions. The extracted DNA was then analyzed via real-time PCR with a TaqMan probe targeting the leptospiral *lipL32* gene [27]. The direct quantification method was applied. The amplification mixture for each sample followed the specifications described by Salgado et al. [27] and Stoddard et al. [28]. A pure culture of pathogenic *Leptospira*, diluted to known bacterial concentrations, served as the positive control for DNA extraction and PCR [29].

This study adhered to the STARD guidelines for reporting diagnostic accuracy studies [30]. Seropositivity, determined with the MAT, was calculated with a modified

TABLE 1 | *Leptospira* species, serogroups, serovars, and reference strains used in the MAT.

Species	Serogroup	Serovar	Reference strain
<i>Leptospira interrogans</i>	Pomona	Pomona	Pomona
	Canicola	Canicola	Hond Utrech IV
	Icterohaemorrhagiae	Icterohaemorrhagiae	Icterohaemorrhagiae
	Autumnalis	Autumnalis	Akiyami A
	Australis	Bratislava	Jez Bratislava
<i>Leptospira borgpetersenii</i>	Sejroe	Hardjo	Ar
	Tarassovi	Tarassovi	Perepeletsin
<i>Leptospira kirshneri</i>	Grippotyphosa	Grippotyphosa	Sag

formula [31], as the number of seropositive animals divided by total number of animals subjected to the MAT (1).

Similarly, the positivity and renal excretion of *Leptospira*, as measured by real-time PCR, were calculated as the number of positive animals divided by the total number of animals subjected to real-time PCR (2).

The 95% confidence intervals (95% CIs) were determined for each proportion [32]. Differences in serogroup frequency and antibody titer were analyzed with the chi-square test with Yates' correction ($p < 0.05$) [33]. Diagnostic performance measures, including the kappa coefficient, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), were calculated in Win Epi software [34].

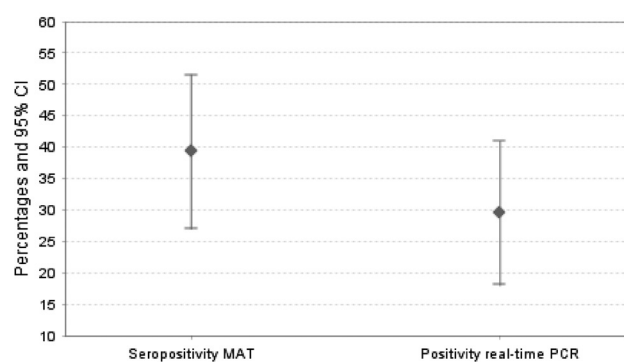
Ethics statement

This research was evaluated by the Institutional Committee on Ethics in Animal Care of the Universidad San Sebastián, Chile (approval number: 14-23).

RESULTS

Detection of seropositive animals, reactive serogroups, and antibody titers with the MAT

Reactivity to pathogenic *Leptospira* was detected in 24 of 61 animals, corresponding to a seroprevalence of 39.3% (95% CI, 27.0–51.5) (Fig 1). The most frequently detected sero-

**FIGURE 1** | Positivity rates for the MAT and real-time PCR in $n = 61$ cattle. Los Lagos region, Chile, 2024.

groups were Tarassovi (52.2%) and Sejroe (41.7%), and a single case of co-agglutination involved Hardjo and Tarassovi (1.2%). No statistically significant differences were observed in serogroup frequency ($p > 0.05$).

The antibody titers responsible for *Leptospira* seroreactivity were 1:800 and 1:200 (33.3% each), followed by 1:400 (25.0%) and 1:1,600 (8.3%). The differences in antibody titer distribution were not statistically significant ($p > 0.05$).

Determination of positivity and renal excretion of pathogenic *Leptospira* with real-time PCR

The positivity rate for real-time PCR (renal excretion) was 29.5% (95% CI, 18.0–40.9; 18/61 animals) (Fig 1). The number of *Leptospira* per milliliter in urine samples varied from 1.07 to 12,500.

Comparison of MAT and real-time PCR results

A relationship was observed between the MAT and real-time PCR results: 75.0% ($n = 18$) of animals with positive samples were identified with both methods, whereas 25.0% ($n = 6$) were positive only with the MAT. No animals with seronegative samples were PCR-positive, whereas six seropositive animals had negative PCR results. A total of 37 animals tested negative with both methods (Table 2). The kappa coefficient of agreement between tests was 0.784 (95% CI, 0.626–0.943).

With a 95% confidence level, real-time PCR demonstrated a sensitivity of 75.0% (95% CI, 57.7–92.3) and a specificity of 100% relative to the MAT. The PPV and NPV were 100% and 86.7% (95% CI = 75.7–96.4), respectively.

TABLE 2 | Results of the MAT and real-time PCR in $n = 61$ cattle in the Los Lagos region, Chile, 2024.

PCR results	MAT results		
	Negative	Positive	Total
Negative	37	6	43
Positive	0	18	18
Total	37	24	61

DISCUSSION

In this pilot study, 61 bovines were sampled to investigate the relationship between seropositivity for pathogenic *Leptospira*, as determined with the MAT, and bacterial shedding in the urine, as detected by real-time PCR. Additionally, the diagnostic utility of both tests for *Leptospira* seropositivity and infection was assessed. The observed seroprevalence was 39.3% according to the MAT, whereas real-time PCR identified a positivity rate of 29.5%.

In contrast to our findings, previous studies in southern Chile, particularly in the Los Ríos region, have reported cattle seroprevalence rates of 59.15% [35], 75.0% [36], and 13.4% [29]. These differences might be attributable to differences in the MAT panel of serovars used [37], as well as environmental and management factors, such as pest control and fencing to prevent contact with other cattle or wildlife [38]. Epidemiological studies on bovine leptospirosis across the Americas have reported varying seroprevalence rates, even within the same country. For example, rates of 67.5% and 64.4% in Mexico [39,40]; 49.3% and 40.5% in Brazil [41,42]; and 16.0% and 34.2% in Colombia [43,44] have been reported. Higher prevalence rates have been observed in tropical climates, where warm temperatures and humidity favor bacterial survival and increase infection risk [38].

MAT analysis revealed a high frequency of serological reactions (seropositivity) for the Tarassovi and Sejroe serogroups. Research on cattle in Chile has frequently reported the Sejroe serogroup, and Hardjo is the most identified serovar [36,45,46]. This finding reflects widespread exposure and serogroup-specific positivity, probably because cattle serve as maintenance hosts for this serovar [47]. Transmission within the same herd or between herds may be an important source of infection. The Sejroe serogroup has also been identified in cattle in other American countries, including Mexico [40], Brazil [41], and Ecuador [48]. In contrast, the seropositivity of Tarassovi is associated with wild animals and pigs, which have been identified as reservoirs [49]. This serogroup has also been identified in cattle in Mexico [39], Uganda [50], Madagascar [51], and Thailand [52].

The detected antibody titers were 1:800 and 1:200 (each observed in 33.3% of cases), followed by titers of 1:400 (25.0%) and 1:1,600 (8.3%). Notably, the sampled animals were not vaccinated and did not exhibit clinical signs of leptospirosis. According to WHO-ILS [25], an antibody titer of 1:100 is sufficient for a positive MAT test result, whereas titers of $\geq 1:400$ indicate active infection. This threshold suggests that some animals in this study might have had active infections. Pinto et al. [53] have reported that leptospirosis in cattle, particularly that due to Sejroe serogroup serovars, is typically asymptomatic, because of host adaptation. However, Soares et al. [54] have noted that asymptomatic infections can also occur with other serogroups. The MAT on paired serum samples remains the

preferred method for detecting IgM and IgG antibodies, and confirming leptospirosis diagnoses, even in animals without clinical signs [37,55]. The asymptomatic seropositivity observed in this study underscores the need for increased awareness of *Leptospira* infection among livestock producers and veterinarians in southern Chile. Preventive measures, such as vaccination, are recommended. Because adaptive immunity in leptospirosis is serotype-specific, vaccination protects against only homologous serovars without cross-immunity [56]. In Chile, available cattle vaccines include serovars Hardjo, Pomona, Canicola, Icterohaemorrhagiae, and Grippotyphosa [57].

In a review by Di Azevedo and Lilenbaum [58], real-time PCR was found to have been used in 38 (37.2%) of 102 reviewed studies, and the *lipL32* gene was the most common target for *Leptospira* detection (applied in 48% of cases). Real-time PCR can detect the disease before antibodies are produced and therefore is a valuable early diagnostic tool, alone or in combination with other tests, for increasing diagnostic accuracy [59]. In the present study, real-time PCR detected a pathogenic *Leptospira* positivity rate and renal excretion rate of 29.5%. The estimated leptospiral concentration in urine samples ranged from 1.07 to 12,500 leptospires per milliliter, thus indicating varying environmental contamination and renal bacterial elimination. Certain species, such as *Rattus rattus*, shed large quantities of leptospires per milliliter of urine, whereas cattle and deer shed fewer bacteria. However, due to the greater volume of urine eliminated compared to rodents, these animals still contribute to environmental contamination and disease epidemiology [60]. A study by Monti et al. [29] on six dairy farms in southern Chile has reported bacterial loads ranging from 30,000 to 44,000 leptospires per milliliter, indicating higher bacterial excretion levels than observed in the present study.

This study identified a relationship between pathogenic *Leptospira* detection via MAT positivity and renal excretion determined by real-time PCR: samples from 75.0% of animals tested positive in both assays. This result supports the complementarity of these diagnostic tests despite their different detection targets: the MAT identifies anti-*Leptospira* antibodies, whereas real-time PCR detects leptospiral DNA [1,4]. Bharti et al. [61] have reported that pathogenic leptospires can be isolated from blood and cerebrospinal fluid samples within the first 10 days after infection, whereas after 14 days, the bacteria are recovered primarily from urine samples. The MAT has low sensitivity in the acute infection phase, because of the absence of a humoral immune response, but achieves optimal sensitivity in the immune phase [62]. *Leptospira* colonizes the proximal renal tubules of reservoirs, thereby resulting in prolonged bacterial shedding in urine [61,63]. Some infected animals in this study were likely to have had longstanding infections detectable by both real-time PCR and the MAT. The sensitivity and specificity of real-time PCR versus the MAT were 75% (95% CI, 57.7–92.3) and 100%, respectively. The high specificity indicated that

real-time PCR can effectively identify healthy animals and accurately exclude individuals without renal bacterial excretion [64]. Although serology is useful for herd diagnosis, it might be inadequate for identifying individual maintenance hosts; consequently, bacterial detection through PCR is required [65]. Therefore, direct diagnostic tests such as PCR have become crucial leptospiral infection diagnostics [66].

Real-time PCR exhibited a PPV of 100% and an NPV of 86.7% (95% CI, 75.7–96.4). These values reflect the test's accuracy in distinguishing true positives and true negatives, which is crucial for predicting disease status according to test results [64]. The PPV and NPV complement sensitivity and specificity estimates for a given diagnostic test [67]. In the present study, a positive real-time PCR result indicated *Leptospira* infection with 100% probability, thus underscoring the test's reliability. Unlike sensitivity and specificity, which remain stable for a diagnostic test, predictive values depend on the pre-test probability: positive predictive values increase with disease prevalence, whereas negative predictive values increase as the prevalence declines [66]. The kappa coefficient between the MAT and real-time PCR of 0.784 (95% CI, 0.626–0.943) indicated substantial agreement [68]. In a recent study in humans, 81% of cases tested on the basis of both serology and PCR were confirmed infections; therefore, the use of both methods is the most effective diagnostic strategy in settings with access to these techniques [69]. Combining the MAT and PCR increases diagnostic accuracy and prevents misdiagnoses, thus facilitating appropriate therapeutic management [70].

The relatively small sample size is a key limitation of this study. However, our findings confirm the complementary nature of the MAT and real-time PCR. The MAT detects antibody titers and identifies the leptospiral serogroup responsible for the serologic reaction [15,16], whereas real-time PCR quantifies bacterial DNA [71], and measures leptospires per milliliter of urine as an indicator of bacterial load.

Future epidemiological studies should include larger sample sizes, particularly in regions where bovine leptospirosis negatively affects livestock reproduction and economic stability, such as southern Chile. Additionally, collecting serial samples over time for both the MAT and real-time PCR would enable tracking of immune response dynamics and bacterial renal excretion patterns.

CONCLUSIONS

This study demonstrated that 75.0% of tested animals yielded positive results with both diagnostic assays, thus highlighting the complementarity between the MAT and real-time PCR findings. Real-time PCR detects *Leptospira* infection, quantifies bacterial excretion in urine, and assesses environmental contamination, whereas the MAT determines seropositivity, antibody titers, and the specific serogroup involved. Serogroup identification

enables tracing of potential infection sources and serological reactivity.

Given their distinct yet complementary roles, these tests should be used in combination to enhance diagnostic accuracy. Implementing both assays when laboratory resources are available would significantly improve the detection and management of leptospirosis in cattle populations.

ACKNOWLEDGEMENTS

To the Veterinary Clinical Hospital of the Universidad San Sebastián, Sede de la Patagonia, Puerto Montt, Chile; to the farmers who allowed their animals to be sampled for this study and to Ms. Gloria Meniconi Ponce, veterinarian, for her help in processing the samples.

CONFLICTS OF INTEREST

None.

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