

***Leptospira interrogans* serogroup Sejroe serovar Hardjo in aborting cows: two herd cases in Sicily (Italy)**

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Received: July 4, 2019

Accepted: March 2, 2020

Abstract

Introduction: The aim of this study was to present two outbreaks of bovine abortion due to *Leptospira* infection in cattle herds located in the northern part of Sicily (Italy). The animals were positive for *Leptospira interrogans* serogroup Sejroe serovar Hardjo in a microscopic agglutination test (MAT). **Material and Methods:** A total of 23 Charolaise cows (farm A) and 75 Limousine bulls and Cinisara and Modicana cows (farm B) were enrolled in this study. The blood samples were collected from all subjects at the following time points: before a cycle of intramuscular treatment with oxytetracycline dihydrate (T0), after 5–6 weeks from the treatment (T1), and every 10 weeks until seronegativisation (T2 in Farm A and T3 in Farm B). A serological test (MAT) was used for the diagnosis of leptospirosis. **Results:** Two samples from farm A (2/23) and 29 samples from farm B (29/75) were positive to *Leptospira interrogans*, serogroup Sejroe, serovar Hardjo in the MAT. *Leptospira* spp. DNA was detected by real-time PCR in the urine sample of one positive cow on farm A, and in placenta and brain samples belonging to one aborted foetus on farm B. **Conclusion:** It is important to use serological and molecular diagnostic techniques complementarily to identify infected individuals.

Keywords: cattle, leptospirosis, microscopic agglutination test, PCR, zoonosis.

Introduction

Leptospirosis is a neglected zoonosis with worldwide distribution that affects humans and a broad range of wild and domestic animals. The causative agents are spirochetes, gram-negative and obligate aerobic bacteria belonging to the genus *Leptospira* and displaying very broad serovar diversity. While the disease is endemic in many countries, it often presents as epidemic outbreaks, causing severe and sometimes fatal disease in both humans (22) and animals (6). In Italy, human infection occurs rarely. Leptospirosis is transmitted mainly through direct contact with the urine of infected reservoirs, as well as through consumption of contaminated water and food (18). Although rodents are considered the main reservoirs of *Leptospira* in rural areas, unlike in urban settings, other domestic and wild animals can play an important dissemination role (15). Infected bovines also constitute an active

reservoir for the spread of the zoonosis, especially for humans in direct contact with infected animals (4, 23). In cattle, leptospirosis is the major cause of reproductive failure, including abortions and stillbirths and weak newborns, and the cause of depression of dairy cattle's growth rate and milk production (7). According to Saito *et al.* (20), there are approximately 300 serovars of *Leptospira* spp., divided into 28 groups. To date, most cases of bovine leptospirosis are attributable to the serovars belonging to the species *Leptospira borgpetersenii*, *Leptospira interrogans*, and *Leptospira kirschneri*, which appear to also be associated with the human disease (14, 23). Cattle are recognised as maintenance hosts of serovar Hardjo, as well as other members of serogroup Sejroe. However, serovars Pomona, Icterohaemorrhagiae, and Grippityphosa can also be linked with bovine infection (7, 8, 10). In a recent study in Italy, serovar Hardjo (serogroup Sejroe) was confirmed as the most prevalent

agent of *Leptospira* infection in cattle (79.6%), followed by serovars Pomona (serogroup Pomona) (14.4%), and Bratislava (serogroup Australis) (3.4%) (21).

Infection by serovar Hardjo generally manifests no clinical signs but abortion is generally ascribed to serovar Pomona. The latter has been isolated from dairy cattle in Italy (11). The seroprevalence detected of the serogroup Australis suggests the adaptation of this *Leptospira* to domestic animals, confirmed also by the isolation of the serovar Lora from cattle in Italy (3, 21). The serogroup Sejroe, to which *L. borgpetersenii* and *L. interrogans* belong, cause a chronic disease in cattle with symptoms more difficult to detect (21), but in other cases it causes an acute form with various signs, including fever, icterus, haemoglobinuria, infertility, abortion, and death (1). Leptospire frequently localise in the kidneys or reproductive organs such as the uterus, oviductus, ovaries, testes, and epididymis, and are shed in the urine for long periods. After clinical recovery animals continue to shed organisms into the environment intermittently in urine and foetal fluids. The source of infection is an infected animal which contaminates pasture, drinking water, and feed by infective urine, aborted foetuses, and uterine discharges (8). An infected neonate can also harbour the infection for several weeks after birth. Another leptospira source may be the semen of an infected bull, and transmission by natural breeding or artificial insemination can occur but is uncommon (1, 9). The infection occurs either directly in contact with urine or infected genital fluids *via* the transplacental and coital route or indirectly through the surrounding environment (water, soil, or contaminated food) (1). The consequences are abortion, infertility, foetus mummification, high natimortality, or reduction in milk production even to the extreme of agalactia.

The gold standard for diagnosis of leptospirosis is the demonstration or culture of the bacterium from urine or renal tissue. The examination of urine samples for the organism probably allows the presence of infection to be demonstrated. Following natural infection with *L. Hardjo*, cattle may shed leptospire in the urine for between 28 and 40 weeks, and thereafter there is a progressive decline in the numbers shed (1). However, this diagnostic technique is problematic to perform routinely, due to the slow growth rate of some *Leptospira* strains and long incubation periods before an isolate is established in the culture. Furthermore, the success of *Leptospira* isolation depends on some experimental factors, such as the freshness and timing of blood and urine samples, and is conditional on sampling taking place before the start of antibiotic treatment. The microscopic agglutination test (MAT) is the most commonly used serological test for the diagnosis of leptospirosis. In animals which survive infection, acute leptospirosis can readily be diagnosed on the basis of demonstrating a rising antibody titre in acute-phase and convalescent patient sera (1, 16). Following infection, the IgM class of antibodies are

first to appear, followed by IgG antibodies, which persist for a long time. The MAT detects both IgM and IgG antibodies and it can be used after 6–10 days from the infection. It is based on the evidence of agglutinates between anti-*Leptospira* spp. antibodies and antigens (pure *Leptospira* spp. strains), using a microscope equipped with a dark field condenser (17). For the detection of the organism in tissue, molecular diagnostic methods such as real time PCR are used, allowing leptospire to be detected in whole blood and urine samples taken during post mortem examination or from live animals. The aim of this study was to present two outbreaks of bovine abortion due to *Leptospira* infection occurring in October 2017 in cattle herds located in the northern part of Sicily (Italy).

Material and Methods

The detection of outbreaks was determined following abortions and fertility disorders in two herds located in the area of the Madonie park in the northern part of Sicily, entirely included in the metropolitan city of Palermo. The park's coordinates are 37°53'N 14°01'E (Fig. 1). On 20 October, serum samples taken from animals on farms A and B with obvious symptoms suggestive of leptospirosis were sent to the Istituto Zooprofilattico of Sicilia (IZS), with the request to carry out the MAT on them for detection of the *Leptospira* antibodies.

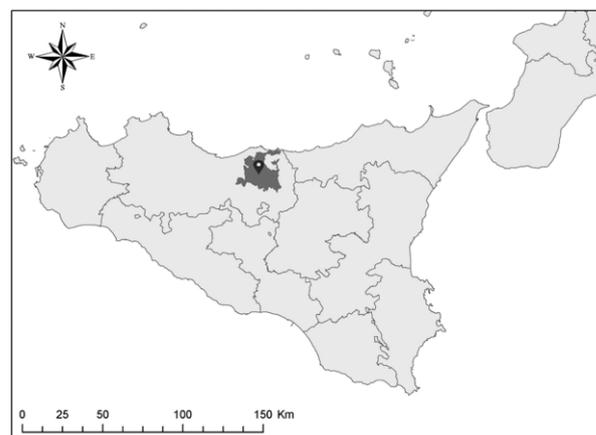


Fig. 1. Geographical area where the farms are located

Animals. Animals enrolled in the study were mainly female (98%) with a mean age of 5.5 years and a mean weight of 500 kg. Farm A consisted of 23 Charolaise cows in a semi-wild cow-calf operation, fed on pasture with their diet improved with forage and concentrates produced on the farm and supplied with water for drinking from wells. The animals were not moved for transhumance. Four cows showed infertility after having aborted in the second half of pregnancy. Clinical findings were depression and fever (40.5–

41.5°C) after abortion. Farm B consisted of 75 Limousine bulls and Cinisara and Modicana cows also in a semi-wild cow-calf operation, fed on pastures located at different altitudes, and with their diet also improved with forage. Water came from artificial wells and natural basins. The animals of this operation had more contact with the various neighbouring farms. The symptom observed in 12 cows in this second herd were the same as in the first herd, with several cases of abortion. Both herds came into the proximity of other animal species such as dogs, wild boar, and pigs. No animals had been vaccinated against leptospirosis.

Samples. In October 2017, 23 (farm A) and 75 (farm B) serum samples were collected, the counts corresponding to all the cows and bulls raised on the farms. The blood samples were collected from all subjects by coccygeal venipuncture using 5 mL tubes with clot activator. The blood collection was performed before a cycle of intramuscular treatment with oxytetracycline dihydrate (Terramicina Long Acting, Pfizer Italia, Latina, Italy), given at a dose of 10 mg/kg of body weight in injections four days apart (this was time point T0), next during follow up examinations after 5–6 weeks from the treatment end (time point T1), and lastly every 10 weeks until seronegativisation (T2 in farm A and T3 in farm B). If antibody titres showed no reduction, at the first follow up (T1) a second antibiotic cycle was performed. Two (2/2) and four (4/29) urine samples were collected from serologically positive asymptomatic cows on farms A and B, respectively. Furthermore, one aborted foetus with placenta was collected from each farm. Blood samples were subjected to centrifugation at 3,000 rpm for 10 min at room temperature; sera were kept at 4°C until use.

Serological test. The strains, selected and provided by the Italian National Reference Centre for Leptospirosis, were grown in liquid leptospiral Ellinghausen–McCulloch–Johnson–Harris (EMJH) culture medium at 30°C for 4–8 days. The panel of antigens was composed of eight serogroups, which are representative of all the serogroups known to exist in the Italian area: *L. interrogans* serogroup Australis serovar Bratislava, *L. interrogans* serogroup Pomona serovar Pomona, *L. kirschneri* serogroup Grippotyphosa serovar Grippotyphosa, *L. borgpetersenii* serogroup Ballum serovar Ballum, *L. interrogans* serogroup Sejroe serovar Hardjo, *L. borgpetersenii* serogroup Tarassovi serovar Tarassovi, *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni, and *L. interrogans* serogroup Canicola serovar Canicola. The EMJH medium was prepared with 2.3 g of *Leptospira* dehydrated base in 900 mL of purified water (Becton Dickinson Italia, Milan, Italy), to which was added pyruvate sodium 10% (1 mL) and glycerol 10% (1 mL) (Sigma Aldrich, Milan, Italy). The pH solution was 7.4 with the addition of HCl 1 M or NaOH 1 M (Merck, Milan, Italy). The medium was sterilised

through a filtration system and was maintained at $\pm 4^\circ\text{C}$. Before utilisation, having reached room temperature the medium had Leptospira Enrichment EMJH added in 100 ml volume (Becton Dickinson Italia). The MAT was performed as described by the OIE guidelines (18). This serological test is based on the agglutination due to the interaction between the antigen and the anti-leptospiral antibodies present in the serum. The antigen-antibody complexes were examined by dark-field microscopy and samples showing titres equal or higher than the MAT cut-off of 1:100 against one or more serovars were considered positive. The endpoint was the dilution of serum that showed 50% agglutination.

Isolation of *Leptospira*. The urine was transported in a selective culture medium, which was EMJH containing 5-fluorouracil at 100 $\mu\text{g}/\text{mL}$ (Sigma Aldrich). In the laboratory, 1 mL of urine was aseptically inoculated in 9 mL of medium diluted 1:10. The isolation procedure was performed as described by the OIE guidelines (18).

Molecular techniques. A real-time PCR was performed on the brains of aborted foetuses, placenta tissue, and urine, based on the detection of the *lipL32* gene present on the external membrane of pathogenic *Leptospira*. The sequences of primers and probe are presented in Table 1.

Table 1. Nucleotide sequences of primers and hydrolysis probe to amplification of *lipL32* gene

Oligonucleotide	Sequence 5'–3'
Primer F	GGTCTTACACAATTTCTTCACT
Primer R	TGGGAAAAGCAGACCAACAGA
Probe	AAGTGAAAGGATCTTTCGTTGTTGC

Reference DNA of *Leptospira interrogans* serogroup Australis serovar Bratislava was used as a positive control, provided by the National Reference Centre for Leptospirosis, Istituto Zooprofilattico Sperimentale of Lombardia and Emilia Romagna and maintained at our laboratories. The reaction mixture was 5 μL of template DNA, 0.4 mM of *lipL32F* and *lipL32R* primers, 0.2 mM of probe ?GATCTTTCGTTGTTGC–BHQ-5'), 1:1 Sso Advanced Universal Probes Supermix (Biorad Laboratories, Segrate, Italy), 2 μL of 1:10 Exo IPC Mix, and 0.5 μL of 1:50 Exo IPC DNA (ThermoFisher Scientific, Rodano, Italy). The final volume of reaction mixture was adjusted to 25 μL with sterile water. The amplification programme was initial denaturation (95°C for 5 min) and 45 cycles of amplification (denaturation at 95°C for 15 s and annealing and elongation at 60°C for 30 s). As a control, a QuantiFast Pathogen PCR Kit (Qiagen, Hilden, Germany) was used.

Results

On farm A, two samples were positive (2/23) for *Leptospira interrogans* serogroup Sejroe serovar Hardjo at MAT before treatment, with a titre of 400 (T0); at T1 the titre was 200 in one subject, which was treated again, and <100 (negative) in the other; at T2 all sera were negative (Table 2).

Table 2. Serum samples of cows on the farm A by microscopic agglutination test (MAT) with cut-off of ≥ 100 (n = 23). * <100 negative samples

Positive samples (n = 23)	Antibody titre	Antibody titre	Antibody titre
	T0	T1	T2
1	400	200	<100*
2	400	<100*	<100*

The real-time PCR showed *Leptospira* spp. DNA in the urine sample belonging to one positive cow, but no DNA was detected in placenta or brain samples. On farm B, 29 samples were positive (29/75) for *Leptospira interrogans* serogroup Sejroe serovar Hardjo at MAT, including 15 samples with high titres (from 800 to 3,200) at T0. At T1, 6 positive samples showed an increased titre, 4 samples showed a reduced titre, 19 showed an unchanged titre, and 2 new positive samples were found (31/75). At T2, 11 samples showed seroconversion, becoming negative. At this time, despite the second cycle of treatment, there were still several positive samples (20/75), 9 of which showed a lower titre than the previous time point and 11 an unchanged titre; no new positive samples were found. At T3, all sera were negative (Table 3). Real-time PCR showed *Leptospira* spp. DNA in placenta and brain samples belonging to one aborted foetus, but no DNA in urine samples. The urine cultures showed no *Leptospira* growth.

Discussion

This study presented two outbreaks of bovine abortion due to *Leptospira interrogans* serogroup Sejroe serovar Hardjo occurring in the metropolitan area of Palermo, in the northern part of Sicily (Italy). As bovine leptospirosis is a herd problem with a dynamic epidemiology, it is important to perform investigations of clinical disease in order to obtain more epidemiological information. Furthermore, various studies are needed to understand and design effective control strategies for this zoonosis, using modern diagnostic tools. The data obtained in this study confirm that *L. interrogans* plays a role in determining leptospirosis infection in cattle reared in Sicily, in agreement with a recent serological surveillance study of the disease on Italian territory (21). Furthermore, the presence of other animals (dogs,

wild boar, and pigs), acting as reservoirs in the cattle pastoral area, may be considered a factor for leptospirosis in these herds contributing directly to disease dissemination. The wild boar is a known animal host of *Leptospira* spp. and is considered a potential source of leptospires, which then infect humans and domestic animals. An increase in the population density of wild boar has been documented in many European countries, including Italy (5, 13, 24), and a consequential increase in potential interactions among wild boar, humans, domestic animals, and other wildlife species could increase the dissemination risk of such a disease (19). We suggest that wild boar, pigs, and dogs within the grazing range should be evaluated for potential risks of transmission that they may pose and that co-grazing (particularly with pigs) should be avoided.

Table 3. Positive serum samples of cows on farm B by microscopic agglutination test (MAT) with cut-off of ≥ 100 (n = 75). * <100 negative samples. ** New positive samples found at T1

Positive samples (n = 75)	Antibody titre			
	T0	T1	T2	T3
1	1,600	800	800	<100
2	100	100	<100*	<100
3	1,600	3,200	1,600	<100
4	800	800	400	<100
5	100	100	<100*	<100
6	400	800	800	<100
7	100	200	<100*	<100
8	100	100	<100*	<100
9	200	200	<100*	<100
10	100	100	<100*	<100
11	800	400	400	<100
12	800	800	<100*	<100
13	1,600	800	400	<100
14	400	800	400	<100
15	100	100	<100*	<100
16	800	800	800	<100
17	800	1,600	1,600	<100
18	400	200	<100*	<100
19	1,600	1,600	800	<100
20	200	200	200	<100
21	100	200	<100*	<100
22	200	200	200	<100
23	800	400	200	<100
24	1,600	1,600	1,600	<100
25	3,200	3,200	1,600	<100
26	800	800	400	<100
27	800	800	800	<100
28	200	200	200	<100
29	800	800	400	<100
30	<100*	100**	<100*	<100
31	<100*	200**	200	<100

MAT and real-time PCR were selected for the diagnosis of leptospirosis. MAT is a laboratory method that has much greater use than PCR as a herd test (17), and recently Tagliabue *et al.* (21) utilised serological data obtained in MAT from the sera of domestic and wild animals to update the epidemiological situation of leptospirosis in Italy. Despite the small number of samples tested, the *Leptospira* DNA detection by PCR in urine samples from asymptomatic cows emphasises that the disease can be disseminated in the environment by viable pathogens excreted through the urinary tract also from positive but clinically healthy animals. However, in this study the growth of leptospire in media was not observed, probably due to some modifications of experimental factors that occurred during the sample handling. The real-time PCR showed *Leptospira* spp. DNA in placenta and brain samples from one aborted foetus from farm B, recognising the agent as the cause of abortions.

Antibiotic therapy plays a major role in reducing the number of infected animals and minimising urinary shedding and cow-to-cow transmission (7). Streptomycin, oxytetracycline, tulathromycin, and ceftiofur are reported to be effective antibiotics for treating leptospirosis (2). In this study, the results of MAT performed before and after the antibiotic treatment with oxytetracycline confirmed that the treatment (two antibiotic administrations four days apart, repeated a second time on farm B) was effective in reducing the antibody titre. In this study no animals had been vaccinated against leptospirosis. Immunisation represents an essential measure for the control of leptospirosis and its adoption is strongly recommended (10). Widespread annual or twice-yearly vaccination in endemic regions using vaccines that include the most common strains circulating in a region seems to be the most effective approach to reduce reproductive problems related to leptospirosis in cattle in the long term. However, although widely used in Europe, livestock vaccination is not yet a regular practice in many countries (12). After an adequate diagnosis by serology and PCR in a urine sample in order to identify shedders and thereby reduce the environmental contamination and transmission to other animals, this outbreak of bovine leptospirosis was successfully controlled with antibiotic treatment. In this study the treated animals showed a decline of antibody titres or even their reduction to zero, allowing us to manage the outbreaks in a relatively short time in compliance with the established national regulations (Italian laws DPR 320/54). This rule provides for the reporting of outbreaks of leptospirosis as an infectious and diffusive disease and the seizure and isolation of infected animals to prevent the spread of pathogens. It is important to underline the need to use serological and molecular diagnostic techniques complementarily to identify infected individuals. Finding only the pathogen or its DNA simultaneously on placenta and foetal tissues allows us to identify the same as the cause

of abortion. In conclusion, facing an outbreak, an integrated strategy for the control of bovine leptospirosis must involve antibiotic treatment and environmental management in order to identify and eliminate the sources of infection (contaminated water and domestic and wild animals as reservoirs) and vaccination, which should be encouraged.

Conflict of Interest Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: The authors received no financial support for the research, authorship, or publication of this article.

Animal Rights Statement: All experimental procedures were carried out in accordance with European legislation regarding the protection of animals used for scientific purposes (European Directive 2010/63).

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