Journal of Microbiological Methods 166 (2019) 105737

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Development of a pooled antigen for use in the macroscopic slide agglutination test (MSAT) to detect Sejroe serogroup exposure in cattle

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ARTICLE INFO

Keywords:
Leptospirosis
MSAT
Bovines
Pará
Amazonia

ABSTRACT

This study aimed to develop and evaluate a pooled antigen for use in the macroscopic slide agglutination test (MSAT) to detect cattle positive for the Sejroe serogroup. To this end, 193 bovine serum samples from different Pará State regions (Amazonia) were subjected to a reference microscopic agglutination test (MAT) for the serological diagnosis of leptospirosis using 11 serovars representing the Sejroe serogroup: Hardjo-prajitno; Hardjo-bovis; Sejroe; Wolffi; Guaricura (Bov.G.); Guaricura (M4/98); Ricardi; Gorgas; Recreo; Polonica and Medanensis. The three most prevalent serovars in the MAT were selected for the development of a pooled antigen for use in MSAT; subsequently, the 193 serum were assessed with the macroscopic slide agglutination test (MSAT) containing the developed antigen. The Kappa test was used to determine the general agreement between the MAT and MSAT results. As a result, of the 193 serum samples, 155 (80.3%) were reactive, and 38 (19.7%) were non-reactive in the MAT; Hardjo-prajitno, Wolffi and Medanensis were the three most prevalent serovars. Of the 193 serum samples tested in the MSAT using the developed pooled antigen, 114 were reactive (59.0%), and 79 (41.0%) were non-reactive; the Kappa coefficient was 0.52 (CI 95%, 0.40–0.63), indicating moderate agreement between the two tests. The MSAT with the pooled antigen including the most prevalent serovars detected bovines with the Sejroe serogroup exposure, mainly in animals with high titters in the MAT, and could be used to screen herds suspected of acute infection by this serogroup in Pará State.

1. Introduction

Leptospirosis in cattle is characterized by reproductive disorders such as infertility, abortions, birth of premature or weak calves and stillbirth, resulting in a great economic impact on the production of these animals (Ellis, 1994; Faine et al., 1999). When leptospires are present in a herd, control becomes difficult because different strains of *Leptospira* may be adaptable to cattle (Pinto et al., 2017), making the cattle reservoirs that maintain the agent in the environment by transmission through the urine (Hashimoto et al., 2017). Although cattle are susceptible to any of the pathogenic serovars of the *Leptospira* genus, strains belonging to the Sejroe serogroup are the most frequently reported in Brazil (Pinto et al., 2016; Pinna et al., 2018; Barbosa et al., 2019).

In Pará State, the Brazilian Amazon region, Negrão et al. (2000) carried out a serological study in cattle from the eastern region of the State and Marajó Island and detected serovar Hardjo as one of the most prevalent; Homem et al. (2001) found the largest number of reactive bovines for the serovar Hardjo in Uruará municipality; Favero et al. (2001) verified that serovars Hardjo and Wolffi were the most prevalent in cattle of 11 municipalities from different State regions; Chiebao et al. (2015) identified bovines reactive for serovar Hardjo in municipalities of all State mesoregions. These studies used the microscopic agglutination test (MAT) as a diagnostic test, revealing that the Sejroe

Abbreviations: MSAT, macroscopic slide agglutination test; MAT, microscopic agglutination test; CI, confidence interval; EMJH, Ellinghausen-McCullough-Johnson-Harris; PBS, *phosphate-buffered saline*; CEUA, Ethics Committee on Animal Use; FMVZ, Faculdade de Medicina Veterinária e Zootecnia; FIOCRUZ, Fundação Oswaldo Cruz; v/v, volume/volume

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https://doi.org/10.1016/j.mimet.2019.105737
Received 12 August 2019; Received in revised form 7 October 2019; Accepted 7 October 2019
Available online 15 October 2019
0167-7012/

serogroup is the most prevalent. Recently, a study showed that the species L. *borgpetersenii* and L. *interrogans,* with genetic similarities to strains of serovar Hardjo, were present in kidney and urine samples from bovine females raised on Pará (Guedes et al., 2019).

The microscopic agglutination test (MAT), in which a panel of live antigens representing the most prevalent serogroups in a specific region is used, is the reference test for the serological diagnosis of leptospirosis; however, it requires specific laboratory equipment and trained personnel for its execution (Faine et al., 1999; Bharti et al., 2003).

The macroscopic slide agglutination test (MSAT) was developed in the late 1950s to facilitate the serological diagnosis of leptospirosis; it is a rapid, practical and accessible test. The MSAT indicates the possible infective serogroup using an antigen in suspension, which may include a pool of up to three concentrated and inactivated serovars. The technique involves the addition of this suspension to the blood serum and then verification of agglutination, similar to the tests used for the serological diagnosis of salmonellosis and brucellosis (Galton et al., 1958; Faine, 1982). The MSAT can be used for the diagnosis of leptospirosis in humans (Sumathi et al., 1997; Brandão et al., 1998) and animals (Solorzano, 1967; Lilenbaum et al., 2002; Lima, 2008). Thus, the aim of this study was to develop and evaluate a pooled antigen for use in the macroscopic slide agglutination test (MSAT) to detect cattle reactive for the Sejroe serogroup.

2. Materials and methods

We used 193 serum samples from bovines from different regions of Pará State, Brazilian Amazon region (Fig. 1). The sera are part of a sample collection held by Laboratório de Zoonoses e Saúde Pública of the Instituto de Medicina Veterinária – Universidade Federal do Pará, Brazil, and were received by the laboratory between 2015 and 2018 for the serological diagnosis of leptospirosis. The samples were from animals belonging to herds with suspected leptospirosis (some with a history of abortion), of different breeds and mixed breeds, containing males and females, used for beef production, of ages ranging from 12 months to 5 years, and without a registered vaccination against leptospirosis.

The serum samples were donated to the Laboratório de Zoonoses Bacterianas of Departamento de Medicina Veterinária Preventiva e Saúde Animal - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil.

Fig. 1. Geographical location of Pará State in Brazil.

Initially, the sera were analysed using the microscopic agglutination test (MAT), performed according to Faine et al. (1999), containing a panel of 11 live antigens found among samples isolated in Brazil and reference samples, all of which were representative of the Sejroe serogroup: Hardjo-prajitno; Hardjo-bovis; Sejroe; Wolffi; Guaricura strain Bov.G. (Santa Rosa et al., 1980); Guaricura strain M4/98 (Vasconcellos et al., 2001); Ricardi; Gorgas; Recreo; Polonica and Medanensis. The serum was considered positive when it was reactive for any serovar at a 1:100 dilution, and the most prevalent serovars were those with the highest number of positive reactions.

The pooled antigen used in the MSAT was composed of a pool of the three most prevalent serovars detected by MAT, and the method to production it, was adapted from that described by Faine (1982) reproduced from Galton et al. (1958). The serovars were cultered separately. The serovars were cultured separately in an initial inoculum of 5 mL in EMJH (Ellinghausen-McCullough-Johnson-Harris) medium, incubated at 30 °C for 5 to 6 days, and then seeded at a ratio of 1/10 (*v*/v) in EMJH medium up to a final 500 mL culture volume for each serovar. The cultures were examined for cell viability and the absence of contamination and then inactivated with the addition of buffered formalin at a final concentration of 0.5% in the culture. Then, centrifugation at 1000 ×*g* for 5 min was performed to eliminate clumps, and the supernatant was centrifuged at 10000 ×*g* for 30 min at 10 °C. The supernatant was removed, and the pellet was resuspended in *phosphate-buffered saline (*PBS, pH 7.4; NaCl 0.137 M; KCl 0.0027 M; Na_2HPO_4 0.01 M; KH_2PO_4 0.0018 M/L) and centrifuged again at 10000 ×*g* for 30 min at a temperature of 10 °C. The supernatant was discarded, and the pellet was resuspended in a buffer for antigen preservation (PBS, pH 7.4, 20% glycerol and 0.5% phenol). Each serovar was passed through a syringe for cell disaggregation and filtered by gauze to remove any extraneous material and then adjusted to an optical density of 0.33–0.35 at 550 nm.

For final mounting of the pooled antigen, equal volumes of the three serovars were mixed in a receptacle, stained with Ponceau S (1%) and stored at 4 to 7 °C for a minimum period of one week for stabilization. The pooled antigen was tested with positive and negative control sera for validation (hyperimmune sera of reference - Royal Tropical Institute, Amsterdam- Netherlands). The positive control sera were specific for the Sejroe serogroup, and the negative control sera specifically not reactive to the Sejroe serogroup but were reactive to other distinct serogroups, such as Canicola and Icterohaemorrhagiae.

The macroscopic slide agglutination test (MSAT) was executed by placing 10 μL of each undiluted serum sample on a glass plate divided into 2.5 by 2.5 cm squares (brucellosis test plate). Then, 50 μL of pooled antigen was added to the serum, and each sample was immediately homogenized with a glass rod. Thereafter, the plate was placed on an orbital shaker at 125 rpm for 4 min at room temperature. The test readout was performed visually, and the samples were considered reactive in the presence of reddish-pink-coloured clumps and non-reactive in the absence of clumps.

Statistical analysis was performed through descriptive statistics using frequency measures. The Kappa test was used to determine the general agreement between the MAT and MSAT results.

This work was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science (Universidade de São Paulo) – CEUA/FMVZ n° 5555210519.

3. Results

Of the 193 serum samples, 155 (80.3%) were MAT reactive for at least one of 11 serovars belonging to the Sejroe serogroup, and 38 (19.7%) were non-reactive. The titers ranged from 100 to 6400, with the three most prevalent serovars being Hardjo-prajitno, Wolffi and Medanensis (Table 1). It was also observed that all reactive samples in MAT were for positive one or more of these three serovars; thus, these serovars were selected for the pooled antigen preparation. The other

Table 1
Frequency of antibody titers found for the 11 serovars of Sejroe serogroup in relation to the number of reactive samples by MAT.

Serovars	Titers								
	100	200	400	800	1600	3200	6400	Total	Total (%)
Hardjo-prajitno	52	57	29	10	–	–	–	148	95.4
Wolffi	24	39	29	13	4	1	–	110	70.9
Medanensis	24	33	28	10	3	1	1	100	64.5
Guaricura (Bov.G.)	29	33	13	1	–	–	–	76	49.0
Guaricura (M4/98)	27	25	11	5	1	–	–	69	44.5
Sejroe	10	6	1	1	–	–	–	18	11.6
Gorgas	12	5	–	–	–	–	–	17	10.9
Ricardi	6	4	–	–	–	–	–	10	6.4
Polonica	2	2	1	–	–	–	–	5	3.2
Hardjo-bovis	3	2	–	–	–	–	–	5	3.2
Recreo	–	–	–	–	–	–	–	0	0

serovars, even those belonging to the Sejroe serogroup, had different reactivity profiles.

In the MSAT of the 193 serum samples, 114 were reactive (59.0%), and 79 (41.0%) were non-reactive. The addition of Ponceau S dye facilitated the visualization of clumps formation in the reactive samples (Fig. 2).

The comparison of the results of the two tests (Table 2) revealed that there were no false positives in the MSAT; however, the number of reactive samples in this test was lower than that in the MAT, especially in samples with low titers (Table 3). The Kappa coefficient was 0.52 (CI 95%, 0.40–0.63), indicating moderate agreement between the two tests.

4. Discussion

The infection of cattle by the Sejroe serogroup, represented mostly by serovar Hardjo, is reported most frequently in serological studies using MAT as a reference test (Sakhaee et al., 2007; Alla et al., 2016; Prajapati et al., 2018); however, other serological assays, such as the enzyme-linked immunosorbent assay (ELISA) (Ngbede et al., 2013; Padian et al., 2015; Derdour et al., 2017) and microsphere immunoassay (Wynwood et al., 2016) can also detect infection. In Brazil, the largest serological study carried out on cattle showed serovar Hardjo to be most prevalent in 21 states (Favero et al., 2001), with reports of strains pertaining to this serovar isolated in the country (Chiareli et al., 2012; Chideroli et al., 2016). Additionally, another

Table 2
Comparison between MAT and MSAT results using the pooled antigen developed.

	MAT			KAPPA
MSAT		Reactive	Non-reactive	TOTAL	**0.52**
	Reactive	114	0	114	**(CI 95%, 0.40–0.63)**
	Non-reactive	41	38	79	
	TOTAL	155	38	193	

Table 3
Number of reactive and non-reactive samples in MSAT in relation to the highest titer found in MAT per sample.

MSAT	MAT[a]								
	Non-reactive	100	200	400	800	1.600	3.200	6.400	Total
Reactive	0	8	44	41	15	4	1	1	114
Non-reactive	38	33	8	0	0	0	0	0	79

[a] Titers for the three most prevalent serovars (Hardjo-prajitno, Wolffi and Medanensis) found for all positive samples on MAT.

study described the whole-genome sequence of the Norma strain (Hardjo) isolated from bovine in a leptospirosis outbreak (Cosate et al., 2015).

Serovar Wolffi was the second most prevalent serovar among the representative Sejroe serogroup serovars. In Brazil, there is a discussion about the use of this serovar in the MAT antigen panel for serological studies in cattle, because cross reactions occur between this serovar and Hardjo (Castro et al., 2008; Cosate et al., 2017; Santos et al., 2018). Despite this evidence, there is only one report of Wolffi isolation from aborted bovine foetuses (Langoni et al., 1999); nevertheless, the characterization of the isolates was restricted to serology by reference antisera, which would not rule out the possibility of a cross reaction with other serovars of the Sejroe serogroup (Faine et al., 1999). In the Brazilian Amazon, although a probable cross reaction cannot be eliminated, the use of the Wolffi serovar on serology seems to increase reactivity to the Sejroe serogroup (Paixão et al., 2016), presenting several reactions much stronger than those to serovar Hardjo (Freitas, 2016).

Medanensis was the third most prevalent serovar in the MAT and has never been used in serological studies for cattle in Brazil. This serovar was first isolated from a healthy dog in Indonesia (Kouwenaar and Wolff, 1929) and is infrequently reported in the literature, consequently when positive reactions for Medanensis are detected may be considered cross reactions with serovar Hardjo (Loewenstein et al.,

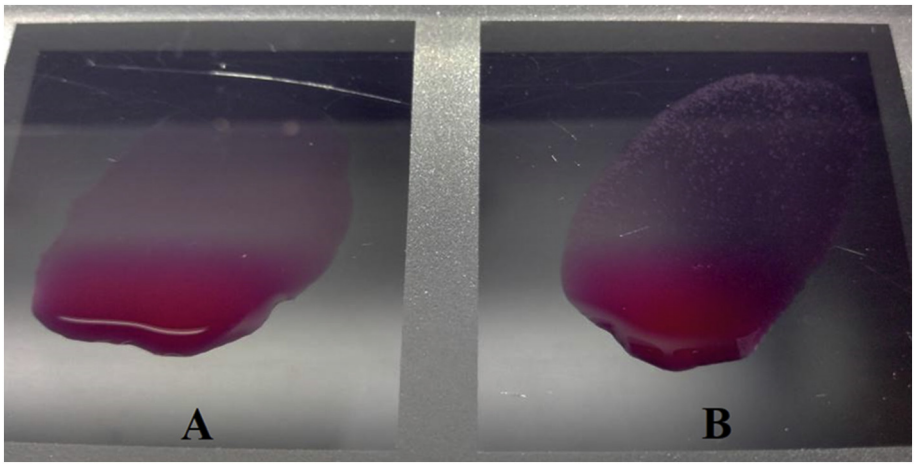

Fig. 2. Validation of the pooled antigen developed for MSAT. On the left-side (A) no clumps (Negative control). On right-side (B) presence of reddish-pink-coloured clumps (Positive control). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2008). More studies are needed to evaluate the viability of including this serovar along with Hardjo and Wolffi in the MAT antigens panel for cattle in Amazonia to expand the coverage of the Sejroe serogroup.

In this study, we used two Guaricura isolates and there was no difference in sera title between the autochthonous strains of these serovar. But the results found for serovar Guaricura disagree in Amazon region with the findings in other regions of the country, where Guaricura is one of the most prevalent serovars alongside Hardjo (Sarmento et al., 2012). Although serovar Guaricura is considered to be prevalent in cattle in the Americas (Barbosa et al., 2019), in Amazonia, our findings corroborate those of Guedes et al. (2019) and suggest that this serovar may not be involved in bovine leptospirosis.

The macroscopic slide agglutination test (MSAT) was originally developed for the serological diagnosis of leptospirosis in humans, mainly for the screening of acute and recent cases of infection (Faine, 1982). The MSAT is as sensitive as the MAT (Chirathaworn et al., 2007) and even more sensitive when a pooled antigen composed of serovars prevalent in the study region is used (Sumathi et al., 1997).

The pooled antigen applied in MSAT is generally not stained; however, the addition of a dye may facilitate the macroscopic visualization of clumps formed in reactive samples (Faine, 1982). Compared to unstained antigen used in the MSAT, Ponceau S-stained antigen in a card test (macroscopic agglutination) used for the serodiagnosis of leptospirosis in humans improved the readability of the test (Bragger and Adler, 1976).

Before the MSAT was developed and disseminated by Galton et al. (1958), a study compared a rapid macroscopic slide agglutination test to the MAT for the detection of Leptospiral Jaundice in humans and reported that blood serum samples that had titers greater than or equal to 200 in the MAT were reactive in the macroscopic agglutination test, whereas samples with titers < 100 were non-reactive in the same test (Starbuck and Ward, 1942). This finding could explain the low sensitivity of the MSAT for the detection of reactive animals with titers of 100 identified by MAT, influencing the Kappa coefficient (0.52) found in the present work. A simulation was performed to assess the agreement between the MAT and MSAT results using only samples with titers ≥200 in the MAT, and the Kappa coefficient found was 0.86 (CI 95%, 0.78–0.95), indicating high agreement between the two tests (data not shown); this finding indicates that the strength of the MSAT reaction increases as the MAT-verified antibody titer increases (Lilenbaum et al., 2002).

In animals, the MSAT has already been explored for the diagnosis of acute leptospirosis in dogs using a commercial antigen produced by Bio-Manguinhos/FIOCRUZ-Brazil and the results were very similar to those found in the MAT (Lilenbaum et al., 2002). For pigs, six antigen suspensions were developed for use in the MSAT to detect anti-*Leptospira* antibodies in animals without clinical suspicion of leptospirosis; the combination of the Pomona and Icterohaemorrhagiae serovars showed the best results, with a sensitivity and specificity of 89% (Lima, 2008). In bovines, an antigen prepared with serovar Sejroe was shown to be efficient for the detection of animals reactive for the Hebdomadis serogroup (Solorzano, 1967), which years after, was reclassified within the Sejroe serogroup (Kmety, 1977). However, another antigen produced with serovar Canicola was unsatisfactory for testing in bovine sera, due to the presence of false-positive reactions when the MSAT was compared to the MAT (Sandhu and White, 1972). In Brazil, this is the first use of an MSAT with a standardized and stained pooled antigen in cattle.

Although the MSAT is an old method, it could be considered an alternative to the MAT for initial screening in acute cases of leptospirosis, allowing a rapid diagnosis and consequently, an early intervention (Brandão et al., 1998). This would be particularly useful in the field where laboratory facilities are not available (Bragger and Adler, 1976), such as in the Amazon region, where there are few laboratories capable of performing the serological diagnosis of leptospirosis.

The MSAT using the developed pooled antigen with the most prevalent serovars detected bovines exposed with the Sejroe serogroup, mainly in animals with high titers in the MAT, and this test could be used to screen herds suspected of acute infection by this serogroup in Pará State.

Acknowledgments

We appreciate Dr. Patrícia Gomes de Souza of Unidade de Diagnóstico de Doenças Bacterianas (DDB) from Laboratório Nacional Agropecuário (LANAGRO/MG) belonging to Ministério da Agricultura, Pecuária e Abastecimento (MAPA) for the donation of serovars: Ricardi, Gorgas, Recreo, Polonica and Medanensis.

MBH, PEB thanked CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) the fellowship. IBG thanked CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the scholarship. This study was financed in part by CAPES, Brasil – Finance Code 001 and by CNPq (420110/2018-6).

Declaration of Competing Interest

None.

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