

Frequency of *Leptospira* spp. in sheep from Brazilian slaughterhouses and its association with epidemiological variables¹

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ABSTRACT.- Da Silva R.C., Costa V.M., Shimabukuro F.H., Richini-Pereira V.B., Menozzi B.D. & Langoni H. 2012. [Frequency of *Leptospira* spp. in sheep from Brazilian slaughterhouses and its association with epidemiological variables.] *Pesquisa Veterinária Brasileira* 32(3):194-198. Departamento de Higiene Veterinária e Saúde Pública, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Campus de Botucatu, Distrito de Rubião Júnior s/n, Botucatu, SP 18618-970, Brazil. E-mail: silva_rcd@yahoo.com.br

Leptospirosis is a worldwide anthroponosis that infects livestock, including sheep as the carriers to other animals and humans. The present study aimed to determine the prevalence of *Leptospira* spp. in sheep from two slaughterhouses in the state of São Paulo, Brazil and its association with epidemiological variables. Serum samples from 182 sheep were evaluated for *Leptospira* spp. antibodies by microscopic agglutination test (MAT). Results indicated 34/182 (18.68%; CI95% 13.70-24.98%) positive serum samples, mainly to the serovar Copenhageni (17/34; 50%; CI95% 33.99-66.01%). Bacterial growth in the Fletcher medium was detected for 13/34 (38.24%; CI95% 23.87-55.08%) animals, and confirmed by Polymerase Chain Reaction (PCR) and sequencing for only two kidney samples from two animals. Thus, treatment and vaccination of sheep, besides rodent control, can be useful to prevent the infection in the studied region since sheep are important *Leptospira* spp. carriers, and its transmission to slaughterhouse workers is mainly through the manipulation of visceral tissues.

INDEX TERMS: *Leptospira* spp., sheep, tissue samples, isolation, PCR, slaughterhouse.

RESUMO.- [Frequência de *Leptospira* spp. em ovinos abatidos em matadouros brasileiros e sua associação com variáveis epidemiológicas.] A leptospirose é uma antroponose mundialmente distribuída que infecta animais de produção, incluindo as ovelhas como carreadores para outros animais e o homem. O presente estudo objetivou determinar a prevalência de *Leptospira* spp. em ovinos de dois abatedouros do estado de São Paulo e sua associação com algumas variáveis epidemiológicas estudadas. Amostras de soro de 182 ovinos foram pesquisadas para a presença de anticorpos para *Leptospira* spp. pela soroaglutinação microscópica (SAM). Os resultados indicaram 34/182 (18,68%; IC95% 13,70-24,98%) amostras positivas, principalmente para o

sorovar Copenhageni (17/34; 50%; IC95% 33,99-66,01%). Crescimento bacteriano no meio de Fletcher foi observado em amostras de 13/34 (38,24%; CI95% 23.87-55.08%) animais, e confirmados pela Reação em Cadeia pela Polimerase (PCR) e seqüenciamento para somente duas amostras renais de dois animais. Assim, o tratamento e vacinação dos ovinos, além do controle de roedores, pode ser útil na prevenção da infecção na região estudada, visto que os ovinos são importantes carreadores de *Leptospira* spp. para o homem, e sua transmissão aos trabalhadores de abatedouros ocorre principalmente pela manipulação das vísceras.

TERMOS DE INDEXAÇÃO: *Leptospira* spp., ovinos, amostras teciduais, isolamento, PCR, abatedouro.

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INTRODUCTION

Leptospirosis is a worldwide infectious, emerging and re-emerging zoonosis caused by a spirochete of the genus *Leptospira* (Bharadwaj 2004), leading to epidemic outbreaks mainly related to specific activities (Trubo 2001, Bens-

chop et al. 2009). Its main reservoirs are pets and livestock, primary hosts essential for the persistence of the infection focus. Humans are accidental or terminal hosts. Transmission through direct or indirect exposure to infected urine is usually associated with a specific season or environmental factors that facilitate this exposure (Lilenbaum et al. 2008). Leptospirosis may be endemic or epidemic for many species, including humans, especially in developing countries and/or in those of tropical climate (Horsch 1999, Bharti 2003, Sacsquispe-Contreras et al. 2003, Martins et al. 2011).

Sheep are considered less susceptible to leptospirosis than other livestock species, such as cattle (Leon-Vizcaino et al. 1987). This anthroponosis can manifest in an acute form in small ruminants, causing pyrexia, anorexia, depression, jaundice, and anemic or hemorrhagic syndromes (Martins et al. 2011).

Leptospirosis causes economical losses in sheep due to abortions. In addition to the reproductive system, leptospires can colonize the kidneys, making sheep potential renal carriers. Thus, the bacteria remain in the environment and sheep may act as a source of infection for other animals and humans (Suepaul et al. 2011). Sheep become infected by contaminated urine from rodents, cattle or wild animals. (Dorjee et al. 2011, Martins et al. 2011, Suepaul et al. 2011).

In Brazil, leptospirosis has been scarcely reported for sheep concerning its etiology and spirochete isolation (Lilenbaum et al. 2007, 2008). Thus, the present study aimed to determine the frequency of *Leptospira* spp. in sheep from Brazilian slaughterhouses and its association with epidemiological variables.

MATERIALS AND METHODS

Study design. This study was carried out in two slaughterhouses (A and B) from São Paulo State. The collected serum samples were sent to the laboratory of the Center for Zoonosis Research (Nupezo), from School of Veterinary Medicine and Animal Science (FMVZ), São Paulo State University (Unesp), Botucatu Campus, SP, Brazil, for serological test.

Tissues from seropositive animals were evaluated for spirochetes by darkfield microscopy (DFM), cultured in Fletcher semisolid medium and assayed for *Leptospira* spp. DNA through molecular techniques. Positive samples were sequenced in the Human Genome Research Center, Institute of Biosciences, São Paulo University (USP), São Paulo Campus, SP. This study was approved by the *Animal Experimentation Ethics Committee*, FMVZ-Unesp, Botucatu Campus, SP (65/2006-CEEA).

Sampling. The software Epi Info 3.5.1 (CDC 2008) was used to determine the minimum number of samples (n), adopting 13.7% as expected prevalence (Lilenbaum et al. 2007), significance level (α) = 5%, 95% confidence level and 6% error limit, which resulted in n=124 animals, adjusted to 182 animals. The present number was determined considering a Brazilian population of 253,500 slaughtered sheep in 2008, and of 8,300 in São Paulo State (3.27%) (Brasil 2009, Sorio & Rasi 2010).

Blood, kidney and liver samples were collected from sheep in two slaughterhouses belonging to the region of Botucatu Municipality, SP, and receiving animals from the southern and southeastern regions of Brazil. Slaughterhouses did not present any planning by sacrifice animals by region. Some days they sacrifice animals from one region, other days from two or three regions.

The sampling was conducted by random selection of the animals in the slaughterhouse by day (one yes, one no). No clinical signs were presented. Blood samples were centrifuged at 1600 x g for 10 min, and the serum was kept at -20°C until the serological test. Epidemiological data such as animal age, sex, breeding system, and origin were also obtained.

Serological test. The presence of anti-*Leptospira* spp. antibodies was determined by microscopic agglutination test (MAT), with 28 different serovars: *Leptospira interrogans* [Australis (Ballico), Bratislava (Jez-bratislava), Autumnalis (Akyiami A), Bataviae (Van Tienen), Canicola (Hond Utrecht IV), Djasiman (Djasiman), Sentot (Sentot), Hebdomadis (Hebdomadis), Icterohaemorrhagiae (RGA), Copenhageni (M20), Pomona (Pomona), Pyrogenes (Salinem), *Hardjo-prajitno* (*Norma*), Hardjo-Mini (Neguita), Hardjo-CTG (local sample from Minas Gerais State, not internationally recognized) (Moreira et al. 1994), Castellonis (Castellón, Wolff (3705)), *L. biflexa* [Andamana (CH11), Patoc (Patoc I)], *L. borgpetersenii* [Javanica (Verdrat Batavia 46/RA 94), Tarasovi (Perepelicin), Shermani (LT821), Hardjo-bovis (L171)], *L. kirshneri* [Cynopteri (3552C), Grippytyphosa (Moska V), Butembo (Butembo)], *L. noguchii* [Panama (CZ214K)] and *L. weilli* [Whitcombi (Whitcombi)]. Animals were considered positive when agglutination occurred at 1:100 or higher dilutions (Brasil 1995).

Bacteriology. Fragments (1.0g) of kidney (marrow) and liver from all positive and 34 negative animals, randomly selected, were separately triturated and homogenized in three tubes containing sterile saline solution each. Before culture, all samples were examined by darkfield microscopy (DFM) (Carl Zeiss, Germany) in order to visualize spirochetes. Then, by using a serial dilution technique (10^{-1} , 10^{-2} and 10^{-3} dilutions), samples were placed into Fletcher semisolid medium (Difco, USA), without antibiotics, added of 300mg.L⁻¹ fluorouracil (Eurofarma, Brazil) (Ris 1974), and incubated at 27°C. For six weeks, they were evaluated by DFM once a week (Brasil 1995, Lilenbaum et al. 2007). Two aliquots (1.0mL) of each homogenized tissue were frozen at -80°C for genomic DNA extraction.

Polymerase Chain Reaction (PCR). The same samples were assayed by PCR. Genomic DNA was extracted from tissue samples by using a commercially available DNA extraction Kit, illustra tissue & cells genomicPrep Mini Spin® (GE Healthcare, USA), and evaluated in a spectrophotometer (GE Healthcare, USA). *Leptospira* spp. DNA was amplified by the primers Lep1 (5'-GGCGGCGCTTAAACATG-3') and Lep2 (5'-TTCCCCCATTGAGCAAGATT-3') targeted to the 16S ribosomal RNA gene from *Leptospira interrogans* (GenBank accession n. X17547.1), in a region with 330 base pairs (bp), as described by Mérien et al. (1992). The process was performed with 25 µL containing 1X PCR buffer (10mM Tris HCl pH 8.0, 50mM KCl), 1.5mM MgCl₂, 200µM dNTP, 10µmol each primer, 10ng purified DNA, and 0.2 U µL⁻¹ Platinum Taq DNA polymerase system (Invitrogen, Brazil). A Mastercycler gradient (Eppendorf, USA) was used with initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 1min, 63°C for 1min and 30s, and a final extension at 72°C for 2 min. PCR products were loaded onto 1.5% agarose gel (Invitrogen, Brazil), stained with ethidium bromide and subjected to electrophoresis in TBE buffer (0.09 M Tris-HCl, 0.09 M boric acid, 2mM EDTA, pH 8.3) for 60 min at 80V (Electrophoresis Power Supply Model EPS 301; GE Healthcare). The amplified DNA fragments were visualized through GelDoc-It™ Imaging System, using VisionWorks® LS Software (UVP, USA).

Sequencing. The amplicons were purified by using ExoSAP-IT (USB) and the sequencing reactions were sent to the Human Genome Research Center, University of São Paulo, Brazil, and carried out on both strands through a MegaBACE™ 1000 (GE Healthcare, USA).

Sequences were analyzed by using the software BioEdit Sequence Alignment Editor 7.0.0 and compared with NCBI database by using BLAST search in GenBank (National Center for Biotechnology Information, Washington, D.C., <http://www.ncbi.nlm.nih.gov/BLAST>).

Statistical analysis. The association between serological results and epidemiological variables was analyzed by Chi-square or Fisher's Exact Test through the software Epi Info 3.5.1, adopting 5% significance (α) (Triola 2005).

RESULTS

Seroreactive sheep accounted for 34/182 (18.68%; CI95% 13.70-24.98%). The serovars Copenhageni (17/34; 50%; CI95% 33.99-66.01%), *Hardjo-prajitno* (4/34; 11.76%; CI95% 4.81-26.74%) and *Hardjo-CTG* (3/34; 8.82%; CI95% 3.20-23.06%) were prevalent, with titers ranging from 100 to 400 (Table 1). No spirochetes were identified by DFM in tissue samples, but 13/34 (38.24%; CI95% 23.87-55.08%) samples presented bacterial growth in Fletcher medium, of which 6/13 (46.15%) were from the kidneys and 7/13 (53.85%) from the liver (Table 2). Twelve animals had positive culture results, since only one sheep had bacterial growth in both tissues. *Leptospira* spp. DNA was confirmed by PCR and sequencing in kidney samples from two animals only (2/34; 5.88%). Both sequences had 100% homology with many *Leptospira interrogans* serovars (GenBank accession n. AE010300.2, FJ154557.1, FJ154552.1, FJ154550.1, FJ154549.1, FJ154545.1, FJ154543.1, FJ154542.1, EU581713.1, AB368966.1, AB368965.1, DQ991474.1, DQ991473.1, DQ991472.1, DQ991471.1, DQ991470.1, DQ991469.1, DQ991468.1, DQ991467.1, DQ991466.1, DQ991465.1, DQ991464.1, AY996793.1, AY996792.1, AY996791.1, AY996790.2, AY631894.1, AE016823.1, FJ154558.1, FJ154569.1, FJ154547.1, AM050586.1, AM050585.1, AM050584.1, AM050583.1, AM050582.1, AM050580.1, AM050571.1, AM050565.1). In addition, tissue samples from the 34 seronegative sheep were negative to the isolation and PCR. A significant association ($P \leq 0.05$) was ob-

Table 1. Frequency of *Leptospira* spp. serovars in 182 studied sheep

Serovar	Positive samples	Percentage (%)	CI95% ^a
Copenhageni	17	50.00	33.99-66.01
Hardjo-Prajitno	4	11.76	4.81-26.74
Hardjo-CTG	3	8.82	3.20-23.06
Hardjo	2	5.88	1.80-19.16
Australis	1	2.94	0.69-14.92
Autumnalis	1	2.94	0.69-14.92
Butembo	1	2.94	0.69-14.92
Djasiman	1	2.94	0.69-14.92
Andamana	1	2.94	0.69-14.92
Sentot	1	2.94	0.69-14.92
Pyrogenes	1	2.94	0.69-14.92
Pomona	1	2.94	0.69-14.92
Total	34	100.00	-

^aCI95%: confidence interval of 95%.

Table 2. Positive results for serology, darkfield microscopy (DFM), bacterial culture, and polymerase chain reaction (PCR)

Register ^a	Serology		DFM	Positive isolation ^b		PCR ^c
	Serovar	Titer		Tissue	Dilution	
A16	Copenhageni	200	-	Kidney	10 ⁻²	-
A20	Copenhageni	100	-	Liver	10 ⁻²	-
A22	Copenhageni	200	-	Kidney	10 ⁻²	-
A24	Copenhageni	400	-	Liver	10 ⁻²	-
A28	Hardjo-Prajitnun	400	-	Liver	10 ⁻²	-
A31	Hardjo-Prajitnun	400	-	Liver	10 ⁻¹	-
A36	Hardjo-CTG	400	-	Kidney	10 ⁻²	-
A64	Hardjo	200	-	Kidney	10 ⁻³	-
			-	Liver	10 ⁻²	-
B1	Sentot	200	-	Kidney	10 ⁻²	+
B32	Hardjo-CTG	200	-	Kidney	10 ⁻²	+
B61	Copenhageni	200	-	Liver	10 ⁻²	-
B67	Copenhageni	200	-	Liver	10 ⁻²	-

^aA = slaughterhouse A; B = slaughterhouse B; ^b Presence of non-specific bacterial growth; ^c Polymerase Chain Reaction (Lep1:Lep2).

served among serological results and breed, municipality and slaughterhouses (Table 3). The last variable presented OR=0.5 (0.2-1.1) for slaughterhouse A (23.9%; CI95% 16.4-33.6%), relative to slaughterhouse B (13.3%; CI95% 7.8-21.9%).

Table 3. Association between epidemiological and serological results for sheep from both slaughterhouses

Variable		Tested	Positive	Positive (%); CI95% ^a	OR (CI95%)	P ^b
Sex	Male	101	18	17.8; 11.6-26.4	1.1 (0.5-2.4)	0.44
	Female	81	16	19.8; 12.6-29.7		
Age (years)	0-1	112	19	17.0; 11.2-25.0	-	0.81
	>1-2	57	12	21.1; 12.5-33.4		
	>2-3	12	3	25.0; 09.1-53.8		
	>3	1	0	00.0; 00.0-00.0		
Breed	Mixed breed	30	9	30.0; 16.7-48.0	-	0.01
	Merin	133	18	13.5; 08.8-20.4		
	Santa Inês	16	5	31.3; 14.2-56.0		
	Other	3	2	66.7; 19.4-93.2		
Breeding system	Hemi-intensive	45	6	13.3; 06.3-26.3	1.7 (0.6-4.3)	0.20
	Extensive	137	28	20.4; 14.6-28.0		
State	RS ^c	137	28	20.4; 14.6-28.0	0.6 (0.2-1.6)	0.20
	SP ^d	45	6	13.3; 06.3-26.3		
Municipality	Santana do Livramento ^c	76	21	27.6; 18.9-38.6	-	0.01
	Uruguaiana ^c	61	7	11.5; 05.7-21.9		
	São Manuel ^d	27	4	14.8; 06.1-32.7		
	Duartina ^d	15	0	00.0; 00.0-00.0		
Slaughterhouse	Itapira ^d	3	2	66.7; 19.4-93.2	0.5 (0.2-1.1)	0.05
	A	92	22	23.9; 16.4-33.6		
	B	90	12	13.3; 07.8-21.9		

^a CI95% = confidence interval of 95%; ^b P = P value to α , 5%; ^c RS = State of Rio Grande do Sul; ^d SP = State of São Paulo.

DISCUSSION

The prevalence identified in this study was higher than those observed by Rocha et al. (1998) (175/5298, 3.3%) in Portugal; Zamora et al. (1999) (36/329, 5.7%) in 11 selected sheep farms from Chile; Ciceroni et al. (2000) (19/313, 6.1%) in 26 localities from Alto Adige, Austria; Cerri et al. (2003) (132/1088, 12.1%) in labs from Italy; Dorjee et al. (2008) (157/2758, 5.7%) in slaughtered sheep from New Zealand; and Lilenbaum et al. (2009) (40/292, 13.7%) in sheep flocks from Rio de Janeiro, Brazil.

Sheep serum samples presented high prevalence to the serovars Copenhageni, Hardjo-Prajitno and Hardjo-CTG, different from those observed by Rocha (1998), reporting the serovars Canicola, Pomona and Cynopteri. The crossed reaction between Hardjo-Prajitno and Hardjo-CTG can occur and must be considered a possibility in this study. These serovars are highly important to herbivorous species, causing abortion (Suepaul et al. 2011). Most of positive animals (28/137, 20.4%) were bred extensively. This value is referent only to the animals bred extensively (reagents/total of animals bred extensively, percentage of reagents). In this case, other herbivorous reservoirs can keep the bacteria in the environment, acting as a source of infection for these sheep. The prevalence in extensive breeding system is present in the south region of Brazil (Herrmann et al. 2004), mainly in Santana do Livramento (21/76, 27.6%) and Uruguaiiana (7/61, 11.5%) municipalities.

Once infected, both male and female sheep can present leptospires in the renal tissue, and reproductive system (Leon-Vizcaino et al. 1985, Lilenbaum et al. 2008), as experimentally observed for the serovars Hardjo-bovis (Farina et al. 1996) and Hardjo (Cerri et al. 1996), emphasizing the high importance of sheep as a carrier of these serovars. Lilenbaum et al. (2009) observed a high frequency of the serovars Sejroe (17/40, 42.5%), Shermani (11/40, 27.5%), Grippotyphosa (4/40, 10%), Icterohaemorrhagiae (4/40, 10%) and Autumnalis (3/40, 7.5%), which are common in humans, dogs and wild rodents. Besides serology, those authors detected positive results by DFM and PCR in four cases only. No sample was positive in the bacteriological culture of urine from sheep, since the cultivation of leptospires from urine is difficult and time-consuming. In addition, the culture may present low sensitivity due to inherent methodological difficulties, including fastidiousness regarding growth in an artificial medium, contamination and intermittent bacterial shedding (Cerri et al. 1996, Lilenbaum et al. 2007). The combined use of MAT as a screening test followed by urine PCR for leptospiral DNA direct detection was adequate to identify carriers among goats and sheep (Lilenbaum et al. 2009).

Epidemiological factors can contribute to keep the bacteria in the environment. The extensive breeding system is an important factor to be considered (Herrmann et al. 2004), although it was not significant in this study ($P>0.05$). Lacerda et al. (2008) observed that the bacterial burden in *Leptospira* infection during periods of normal or high rainfall may be overestimated in the northeast region of Brazil. Regarding to the breed, significative association ($P\leq 0.05$)

was observed, presenting higher prevalence to mixed breed and Santa Inês than the others, all bred in São Paulo State. These two breed are usually bred as hemi-intensive breed system, mainly in São Paulo State, and located in a region with a high pluviometric index, which favor the occurrence of leptospirosis. Analyzing the municipalities, significative association ($P\leq 0.05$) was observed, mainly in Itapira, SP (2/3, 66.7%) and Santana do Livramento (21/76, 27.6%). Itapira presented low number of samples assayed, which compromises the significance of the analysis. In other way, Santana do Livramento, RS, present high pluviometric index and the possibility of sheep-cattle mixed breeding, possible source of infection for sheep.

Zakeri et al. (2010) identified by nested PCR *Leptospira* spp. DNA in 13/75 (17.33%) serum samples from sheep in Iran, of which 11/13 (84.6%) were *L. interrogans* and 2/13 (15.4%) were *L. wolffi*. Leptospirosis is an occupational and recreational disease. Farmers and slaughterhouse workers can be infected by handling animals and their blood and tissues and not using personal protective equipment, respectively. Lesions in the hands are important entrance points of this bacterium into the host. *L. interrogans* serovar Hardjo was found in the kidneys of three seropositive slaughtered ewes but not in their uterus or salpinges (Cerri et al. 1996).

Two animals (B1 and B32) had positive samples isolated from the kidneys, according to DFM and PCR, corroborating the observations of Silva et al. (2007), in Pelotas Municipality, Rio Grande do Sul State, Brazil, that reported for the first time the isolation of *L. noguchii* serovar Autumnalis from one kidney from sheep, to the 16S rRNA gene sequence.

Dorjee et al. (2008) observed high titers to the serovars Hardjo-bovis (33%) and Pomona (4%), also common in herbivores and rodents, respectively. Isolation was obtained from kidneys of 8/32 (22%, positive to Hardjo-bovis), 1/6 (17%, positive to Pomona) and 5/499 (1%, seronegative) sheep. Those authors concluded that the isolation from seropositive animals presented 21.7 higher probability to be positive than that from seronegative ones. That study evidenced a definite risk due to the occupational exposure of workers from a sheep-only slaughterhouse to the two most pathogenic *Leptospira* spp. serovars in New Zealand.

The present study also shows a significant difference ($P\leq 0.05$) between both slaughterhouses based on sheep seropositive samples. Slaughterhouse A, received more animals from RS than SP and had 0.5 higher probability to present a positive animal than slaughterhouse B. Thus, it was in contact with the slaughterhouse workers, which represents the possibility of risk for both slaughterhouses A and B, mainly in the former, where a worker became infected by this bacterium due to sheep tissue handling. These data confirm the findings emphasizing the importance of leptospirosis as an occupational disease, such as the results obtained by Benschop et al. (2009), who detected 23/242 (9.5%) seropositive workers in a sheep slaughterhouse from New Zealand, suggesting a significant exposure of workers to leptospirosis from sheep in the studied slaughterhouse.

Based on the increasing consumption of ovine meat in the last years, with world exportations of 841,862 tons of

fresh meat until 2002, these animals require special attention regarding zoonosis control (Bharadwaj 2004).

Prescott (1992) detected positivity to leptospirosis in 11/63 (17.5%) veterinarians (ten to Bratislava and one to *Icterohaemorrhagiae*) and 11/92 (12.0%) slaughterhouse workers (eight to Bratislava and three to *Icterohaemorrhagiae*), suggesting that Bratislava is the most important pathogenic leptospiral serovar for these occupational risk groups. The importance of this serovar in domestic animals has been identified only in the recent years.

Sheep treatment and vaccination, as well as control of rodents, can result in the control of leptospirosis in the studied region, especially for the safety of slaughterhouse workers. The adoption of individual protective equipments (IPE) may help, but alerting these people is the most important measure to assure the correct use of IPE. Sheep are important *Leptospira* spp. reservoirs for humans and other domestic animals and can be transmitted to slaughterhouse workers mainly by the handling of blood and visceral tissues like kidneys and liver without IPE or when it is inadequately used.

Thus, preventive actions directed to sheep, recognized as reservoirs of leptospires, are extremely necessary in slaughterhouses, since leptospirosis is one of the most important emerging and re-emerging zoonosis.

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