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SEROEPIDEMIOLOGICAL DETECTION OF ANTIBODIES AGAINST *LEPTOSPIRA* spp USING MICROSCOPIC AGGLUTINATION TEST IN URMIA COWS AND SHEEP

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The study was designed to determine the level of incidence, titer and various serovars of leptospira in 203 cows and 166 sheep at Urmia abattoir in 2011. Blood samples were collected during the slaughter of animals and sera were separated to evaluate the serological reaction to Leptospira spp by Microscopic Agglutination Test (MAT) using live antigens representing Leptospira interrogans serogroups: pomona, grippotyphosa, canicola, hardjo, icterrohaemoragiae, and ballum. Overall, 36% of cows and 19.3% of sheep including 33.8% of bulls, 40.5% of female cows, 18.3% of rams and 25% of ewes had a positive reaction to at least one of the leptospira serovars. The most prevalent serovars in cows were pomona (22.7%), grippotyphosa (13.8%), and hardjo (8.4%), and in sheep were grippotyphosa (66.7%), pomona (26.2%) and canicola (7.1%). Other serovars were not detected in cows and sheep. The most prevalent serological titers of 1:100 and 1:200 in cows was 18.2% and 26.6%, and for sheep were 13.5% and 8%, respectively, and of 1:400 in sheep was 2.3%. Cows with a positive reaction to one, two and three serovars were 28.6%, 5.9%, and 1.5% and sheep positive to one and two serovars were 13.3% and 6%, respectively. Age comparison in seropositive cows and sheep showed a significantly increased infection ($p<0.05$) from young to adult ruminants, while no differences were seen regarding gender. The main mixed serovars were between grippotyphosa/pomona, grippotyphosa/canicola and canicola/pomona. The gender comparison of the serovars' distribution revealed that the pomona and grippotyphosa were predominant among other leptospiral serovars in cows and sheep, respectively. In conclusion, the rate of leptospirosis in Urmia cows was about 2 fold in sheep. The most current serovars in cows and sheep were pomona and grippotyphosa, respectively. The majority of animals was infected with one serovar, but polyserovars, are also possible. The highest titer (1:200) was observed in cows and 1:400 in sheep. There was no gender difference, but age was significant between cows and sheep. Finally, leptospirosis as a zoonotic disease must be seriously considered in Urmia cows rather than in sheep, and

therefore, a serious effort must be made to reduce the rate of serological infection and the risk of public health, as well.

Key words: cows, grippotyphosa, Leptospirosis, serology, sheep, MAT, pomona

INTRODUCTION

Leptospirosis is the most prevalent worldwide zoonosis, affecting a wide range of mammals including ruminants, equines, rodents, and human. The disease is caused by pathogenic *Leptospira* introgans species, and occurs from a subclinical infection to a severe syndrome with high mortality rate. Leptospirosis involves public health risk, as well as economic losses in the livestock production industry due to decreased milk yield, abortion, stillbirth, weak calves, weight loss, reproductive complications and occasionally death. Furthermore, the heavy costs of treatment, control and vaccination programs are relevant economic losses of this disease (Radostits *et al*., 2007).

The urine of wild and domestic animals, mainly rodents, small marsupials, ruminants, pigs and dogs which may become asymptomatic carriers, constitute the reservoirs of *Leptospira* in nature (Nally *et al*., 2005). Pathogenic leptospires live in the proximal renal tubules of the kidneys of carriers, although other tissues and organs may also serve as the habitat. They are excreted from the kidney into the urine and may then contaminate the soil and water (Radostits *et al*., 2007). Infections of animals or humans occur via direct contact with urine or indirectly from contaminated water. Humans suffer the acute form of infection, and sometimes with longer term of disease. The severity of the disease may be dependent on the infecting serovars, age, health status and immunological competence of the host (Radostits *et al*., 2007).

Although a number of nonspecific symptoms such as fever, jaundice, abortion, pink stained milk, hemoglubinuria in cows, and stillbirth and agalactia in sheep may be considered to be the clinical signs of the disease (McBride *et al*., 2005), definitive diagnosis relies on the detection of anti-leptospiral antibodies in serum samples (Radostits *et al*., 2007). In other words, the efficacy of leptospira control programs in farm animals relies mainly on the direct identification of carriers (de Nardi Júnior *et al*., 2010; Schonman *et al*., 2010). ELISA (Rajeev *et al*., 2010), PCR (Lilenbaum *et al*., 2009) and Microscopic Agglutination Test (MAT) (Rajeev *et al*., 2010) are the main current serological methods, but MAT still being the “gold standard” is particularly recommended to differentiate the infective serovars from each other (Angela *et al*., 1998).

Human leptospirosis is prevalent only in the northern provinces of Iran, but ruminants such as cows (Schonman *et al*., 2010), buffaloes (de Nardi Júnior *et al*., 2010), and sheep (Tooloei *et al*., 2008; Melo *et al*., 2010) are encountered in many parts of traditional style husbandries (Nasr Esphehani, 2004). Data from several indoor studies by MAT in cows (56.6%) and sheep (17.3%) suggest that the disease is prevalent in the livestock population in many regions (Hajikolaei *et al*., 2007; Zakeri *et al*., 2010), and probably in the northwest of Iran, and Urmia, as

well. The aims were to determine the seroepidemiological detection of disease in cows and sheep, the major serovars and severity (titer level) involved in the ruminants of Urmia, and the plausible roles of gender age parameters on its occurrence.

MATERIAL AND METHODS

Sample collection and preparation

Jugular vein blood was collected from 203 cows (119 male, 84 female) and 166 sheep (142 ram, 24 ewe) immediately after slaughtering the animals at Urmia abattoir in 2011. Before slaughter, the gender of the animals was recorded. The age was determined by the presence of temporary and permanent incisor teeth, starting from temporary, 1, 2, 3 and 4 permanent incisors considered as <1.5, 1.5-2, 3, 4 and >4 years old. The frequency of age distribution in cows was 15, 24, 61, 79, and 24, and in sheep with >1.5, 4 and 4> was 142, 6, and 18, respectively. Samples were stored overnight at 4^{o}C and, subsequently, sera were isolated adopting standard procedure (centrifugation at 3000 rpm for 10 minutes), transferred into 1.5 mL Eppendorf tubes and placed at -20^{o}C to freeze. When the sample collections finished, the frozen sera were transferred to the leptospirosis research centre to assay the MAT.

Microscopic agglutination test (MAT)

MAT was executed basically as described by Turner (1968) with modifications in the leptospirosis research laboratory as follows: A 7–10-day old culture of *L .interrogans* in liquid medium was used as the antigen. The density of leptospires was assessed using a counting chamber (Petroff-Hausser USA) and adjusted to 2×10^{8} leptospires/mL. The six reference serovars of *L. interrogans* including *hardjo*, *pomona*, *icterohaemorrhagiae*, *grippotyphosa*, *canicola* and *ballum* were used as live antigens.

All sera were serially diluted with phosphate buffer solution in a micro titer plate (Greiner), starting from 1:50 dilution, using two fold dilution (1:100, 1:200 and 1:400). Ten μL of diluted serum was then added to 10 μL of respective antigen on a glass slide and placed in a petri-dish with moist paper to avoid drying, and incubated at 30^{o}C for 90 minutes. Finally, the slide was examined under dark-field microscope (Olympus BX50). One antigen control and two standard serum controls (positive and negative) were used each time. Titers of ≥ 1:100 were considered positive. The endpoint titer was determined as the highest serum dilution showing agglutination of at least 50% of the leptospires. Data were analyzed by $SPSS_{13}$ statistical program and Chi-Square was carried out to realize the differences between parameters.

RESULTS

Table 1 shows the frequency and percentage of male and female seropositive to *Leptospira* spp in cows and sheep at Urmia abattoir. Overall, 36%

of cows including 32.8% bulls, 40.5% females, and 19.3% of sheep including 18.3% rams and 25% ewes were positive to at least one serovar.

Table 1. Frequency and percentage of female and male seropositives to *Leptospira* spp in Urmia

Animals	Females			Males		
	Frequency	Seropositive	Percentage	Frequency	Seropositive	Percentage
Cows	84	34	40.5%	119	39	32.8%
Sheep	24	6	25%	142	26	18.3%

No significant differences between infected males and females in cows and sheep

The frequency and percentage of each leptospira serovar detected in cows and sheep is shown in Table 2. Positive serological reaction to serovars in cows was *pomona* 18.2%, *grippotyphosa* 13.8%, and *hardjo* 8.38%, and in sheep was *grippotyphosa* 16.87%, *pomona* 6.62%, and *canicola* 1.81%. None of the samples revealed *icterohaemorrhagiae* and *ballum*. *Pomona* and *grippotyphosa* were the most dominant serovars in cows and sheep, respectively.

Table 2. Frequency and percentage of infected males and females to various lepotospira serovars

Serovar(s)*	Males		Females		Total		Percentage in positives
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	
Cows							
Pomona	23	19.3%	23	27.4%	46	18.2%	63
Grippotyphosa	12	10.1%	16	19.1%	28	13.8%	38.36
Hardjo	13	10.9%	4	4.8%	17	8.38%	23.29
Sheep							
Grippotyphosa	23	16.2%	5	20.83%	28	16.87%	87.5
Pomona	9	6.34%	2	8.33%	11	6.62%	34.38
Canicola	3	2.11%	----------	---------	3	1.81%	9.37

Icterohaemorrhagiae and *ballum* was not found.

End point titers of 1:100 and 1:200 in cows were 18.23% and 26.59%, and in sheep were 14.46% and 6.63%, respectively, but an additional titer of 1:400 observed in sheep was 2.41%. The most frequent serological titers were 1:200 in cows (26.59%) and 1:100 in sheep (14.46%).

Table 3 shows the frequency and percentage of multiple seropositives to leptospira in cows and sheep. Although the majority of animals react to one serovar, some also showed reactions against 2 or 3 serovars. In the majority of the positive samples, *gripotyphosa* and *pomona*, *gripotyphosa* and *canicola*, and

pomona and *canicola* were concurrent. The differences (Chi-Square test) between males and females seropositive to *Leptospira* spp in cows and sheep were not significant.

Table 3. Frequency and percentage of mixed serovars detected in cows and sheep

Serovars	Cows			Sheep		
	Frequency	% in total	% in seropositive	Frequency	% in total	% in seropositive
One serovar	58	28.57%	79.45%	22	13.25%	68.75%
2 serovars	12	5.95%	16.44%	10	6.02%	35.5%
3 serovars	3	1.48%	4.11%	0	0	0

The frequency and percentage of age distribution in leptospira seropositive cows with <1.5 years was 5 (6.85%), 2 years old was 11 (15.1%), 3 years old was 20 (27.4%), 4 years old was 30 (41.1%), and 4> years old was 7 (9.6%), and in sheep with <1.5 years was 26 (18.3%), 4 years old was 1 (16.7%), and 4> years old was 6 (27.7%).

DISCUSSION

The rate of infection in cows (36%) was nearly 2 fold higher in sheep. This result was lower than previously recorded for Urmia ruminants (Zinali *et al*., 2000), but slightly greater than reported from the neighboring province in East Azarbaijan (Shoaie 1995; Tooloei *et al*., 2009). The results of the present study suggest that the incidence of leptospirosis in Urmia ruminants is, for the most part, higher than in other provinces of Iran. The rate of infection was in Iran 17.3% (Hajikolaei *et al*., 2007; Tooloei *et al*., 2008; Zakeri *et al*., 2010), India 14.8% (Savalia and Mahendra, 2008), Tanzania 30.3% (Schonman *et al*., 2010), Nigeria 17.7% (Agunloye 2002), Canada 59.1% (Kingscote 1985), and Brazil 46.9% (Lilenbaum *et al*., 2009). This information shows the widespread infections in Iran and the world, with the highest infection in cows and the lowest in sheep (Talebkhan *et al*., 2003), therefore, this should be taken into account in disease control programs. Leptospirosis was also reported in goats (15.4%), buffaloes (14.6%), horses (14.3%), and camel (13.4%), with the highest rate in donkeys (17.6%) (McBride *et al*., 2005; Vilale *et al*., 2005; Zakeri *et al*., 2010).

Distribution of the reported leptospira serovars found throughout the world indicates that *pomona* and *gripotyphosa* (Kingscote 1985; Meenak and Chella, 2008) were the main serovars as demonstrated for cows and sheep in this study too, however, *icterohemorhagiae*, *harjo*, and *canicola* were reported in many other locations (Schonman *et al*., 2010; Hajikolaei *et al*., 2007). The variation in serovars could be related to the frequency of the samples tested in different studies, or changes in the serovars by the matter of time i.e. conversion from *gripothyphosa* to *harjo* within 10 years in certain places (Zinali *et al*., 2000), and finally, close contact with rodents as a reservoir of *gripothyphosa* (Abdollahpour *et al*., 2009).

Variation in the outbreaks of serovars in many parts of the world is approved. Although in this study *pomona* and *gripothyphosa* were the main serovars and greater than *harjo,* the differences among cows and sheep were not significant The presence of infected animals to leptospira and determination of the responsible serovars, in spite of any vaccination history, will help the control and prevention strategies, as well as the increase in public health programs.

There is no reliable evidence that the gender of an animal can have an effect on disease as resulted in this study and both sexes have equal sensitivity to leptospirosis (Agunloye, 2002), even though the age of animals revealed significant differences and could be called a predisposing factor in the occurrence of the disease. This result is in accordance with findings in Iran and other countries that seropositivity to leptospirosis increases when the animals become adult (Hassanpour *et al*., 2008; Talebkhan *et al*., 2003). In this regard, female cows aged 3 to 4 years and ewes over 4 years old are more susceptible than males and young animals (Hajikolaei, 2010). This claim confirms the clinical signs of abortion and mastitis in leptospirosis which can occur in females and adults cows (Ellis *et al.*, 1994; Erdogan *et al.*, 1993).

The majority of cows (79.5%) and sheep (68.7%) were infected with at least one serovar, but 2 and 3 serovars was also observed. This is supported by other authors who have reported up to 5 serovars in animals (Hassanpour *et al*., 2008; Zeinali *et al*., 2000; Nasr Esphahani, 2004; Tooloei *et al*., 2008). Incidentally, *gripotyphosa* and *pomona*, *gripotyphosa* and *canicola* and *pomona* and *canicola* were found together, meaning that simultaneous infection is possible in animals. In spite of increasing health status during the last few decades, it is clear that the incidence of leptospirosis in Urmia and other provinces has noticeably increased. The reasons could be related to the problems in the clinical diagnosis of leptospirosis due to various clinical types, thus, serological detection would be valuable in the detection of the disease.

The endpoint titers of 1:200 in cows and 1:100 in the majority of sheep in this study were lower than reported from 1:400 to 1:1600 by other authors (Hassanpour *et al*., 2006). This shows that the rate of infection in Urmia ruminants and specifically in sheep is low, therefore, from the epidemiological point of view this would be useful in the control procedures, resulting in the prevention of disease in this region. The comparison of the infected animals revealed that *pomona* in cows and *gripotyphosa* in sheep were the main serovars as reported by Radostits *et al.* (2007) and Levett (2001), too. However, literature shows that *icterhemorrhagiae* was the most widespread serovar in Iran (Talebkhan *et al*., 2003) and *gripotyphosa* in Urmia ruminants (Zeinali *et al.*, 2000), which has now changed to *pomona* in cows.

The main and most reliable serological detection test of leptospira serovars following 15 days after infection in human and animals was known as MAT (Perret *et al*., 2005), because this method is based on using live leptospira serovars, and therefore, it is favorable and more accurate than other current tests. In the case of a lack of live serovars, ELISA (Cousins *et al*., 1991), PCR (Vitale *et al*., 2005) and FA (Rajeev *et al*., 2010) would be valuable in the diagnosis of the disease. Some have recommended mixed tests of MAT and PCR as a screening test for diagnosis

and eradication of leptspirosis (Lilenbaum *et al*., 2009). In conclusion, seropositivity to leptospirosis in Urmia cows was more than in sheep in that *pomona* and *grippotyphosa* were the main serovars in cows and sheep, respectively. Infection to polyserovars was possible among ruminants. Titer 1:200 in cows and 1:100 in sheep was mostly visible. No gender difference was observed, but age impact, mainly at 4 years old was significant. Leptospirosis as a zoonotic disease must be seriously considered in Urmia cows and sheep, and therefore, a concentrated effort must be made to reduce the rate of disease and the risk of public health as well.

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SEROEPIDEMIOLOŠKA DETEKCIJA ANTITELA PROTIV *LEPTOSPIRA spp* MIKROSKOPSKIM AGLUTINACIONIM TESTOM KOD KRAVA I OVACA U REGIONU URMIA

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SADRŽAJ

Ova ispitivanja su sprovedena sa ciljem da se utvrdi stepen zastupljeniosti, titra antitela i različitost sero-varijeteta *Leptospira spp* kod 203 krave i 166 ovaca u regionu Urmia na lokalnoj klanici tokom 2011. godine. Uzorci krvi su prikupljani prilikom klanja životinja i izdvajan je krvni serum radi izvođenja seroloških proba mikroskopskim aglutinacionim testom (MAT) antigenima tipičnim za serogrupu

L. interrogans: *pomona, grypotyphiosa, canicola, hardjo, icterohaemoragia i ballum*. Ukupno je 36% krava i 19,3% ovaca ispoljavalo pozitivnu reakciju na najmanje jedan od ispitivanih antigena. Kod krava je bilo najviše reakcija na serovarijetet *pomona* (22.7%)*, grypotyphiosa* (13,8%) i *hardjo* (8,4%) a kod ovaca na *grypotyphiosa* (66,7%), *pomona* (26.2%) i c*anicola* (7,1%). Reakcije na druge serovarijetete nisu utvrđene. Najčešće registrovane vrednosti titra su iznosile 1:100 i 1:200 kod 18,2% i 26,6% krava i kod 13,5 i 8% ovaca respektivno. Kod 2,3% ovaca utvrđene su vrednosti titra od 1:400. Zastupljenost krava koje reaguju na jedan, dva ili tri serovarijeteta je bio 28,6%, 5,9%% i 1,5%. Ukupno je 13,3% ovaca reagovalo na jedan antigen, a 6% na dva. Razlike vezane za pol životinja nisu utvrđene ali se kod starijih grla zapažao veći broj pozitivnih jedinki ($p<0,05$). Najčešći mešoviti serovarijeteti su bili: *grypotyphiosa*/*pomona, grypotyphiosa*/*canicola* i *canicola*/*pomona hardjo.* Zastupljenost pozitivnih grla ovaca je bila dva puta veća nego kod goveda u regionu Urmia a najzustupljeniji serotipovi kod krava i ovaca su bili *pomona* i *grypotyphiosa* respektivno. Većina pozitivnih jedinki je reagovala na jedan sero-varijetet ali je takođe moguća i mešovita infekcija. U regionu Urmia je neophodna stalna kontrola ove opasne zoonoze radi zaštite zdravlja ljudi i životinja.