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***Leptospira* spp. and *Brucella ovis* seroprevalence in sheep:
preliminary results of one year surveillance program**

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ABSTRACT. In order to assess the diffusion of brucellosis by *Brucella ovis* and leptospirosis in sheep flocks in Tuscany, 410 blood samples were collected from males of 76 breeding farms from January to December 2015. All sera resulted negative for *B. ovis*. A percentage of 17.80% sera was found positive for *Leptospira* spp. Among all breeding farms, 34.21% resulted positive. The most represented serovars were Pomona (6.34%), Hardjo (4.14%), Grippotyphosa (3.17%) and Bratislava (1.70%). The highest antibody titers were detected for serovar Hardjo (1:25600) and serovars Pomona and Grippotyphosa (1:1600). These results confirm the role of sheep as maintenance host for serovar Hardjo and highlight the spreading of serovar Grippotyphosa in the study area. Constant field investigation, especially on farm animals, could be useful to determine trends and diffusion of some occupational re-emerging diseases, such as leptospirosis.

Keywords: *Brucella ovis*, *Leptospira* spp, sheep, serology, Tuscany.

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INTRODUCTION

Brucellosis caused by *Brucella ovis* is a non-zoonotic infection characterized by a clinical or subclinical disease in sheep, subfertility in rams due to epididymis, testicle and accessory sexual glands alterations and, less frequently, placentitis and abortions in ewes. Infected ewes may excrete *B. ovis* through vaginal discharges and milk (López et al. 2006). Brucellosis transmission occurs through passive venereal infection or direct contact, in this case the ram role as an infection spreader is decisive. Rev 1 vaccination program could have contributed to the reduction of the occurrence of *B. ovis* (De Bagues et al., 1995). Since several years vaccination is no longer mandatory in Italy; this could represent a promoting factor for *B. ovis* spreading for which an active serological surveillance program is not planned.

Ovine leptospirosis is an underdiagnosed disease, usually silent, and its effects on livestock are often underestimated (Martins and Lilenbaum, 2014). Even if sheep are usually considered less susceptible to leptospirosis than other livestock species, reproductive disorders and failures due to *Leptospira* and serological positivity have been reported in several countries including Italy (Ciceroni et al., 2000, Cerri et al., 2003, Melo et al., 2010, Tonin et al., 2015). Rarely, leptospirosis in small ruminants may occur in an acute form characterized by anorexia, fever, depression and occasionally anemic or hemorrhagic syndromes (Adler and de la Pena Moctezuma, 2010), but most frequently ovine leptospirosis occurs in a chronic form presented with subfertility, neonatal deaths, abortions, and decreased milk yield, causing substantial economic losses for farmers (Lilenbaum et al., 2009).

On the basis of these observations, considering the presence of numerous sheep farms in Tuscany, a serological survey on *Leptospira* and *B. ovis* prevalence was carried out on the male population of some of the breeding farms involved in the national brucellosis surveillance program (DGR 1204/2009), from January 2015 to December 2015.

MATERIAL AND METHODS

From January 2015 to December 2015, blood samples of 410 rams were collected. All subjects were adult, used as breeding animals and no symptoms were

observed at sampling time. Overall, 76 farms were investigated. Fifty-four were located in the northeast of Tuscany, 16 in the southeast and 6 in northwest area of the region. Farms located in the north raised mainly Massese sheep herds of 30 up to 100 animals, while farms located in the south raised Sarda sheep with herds of more than 100 animals. Blood was collected during the routine surveillance program for *Brucella* spp..

The obtained sera were tested for the presence of *B. ovis* and *Leptospira* antibodies.

For the detection of *B. ovis* antibodies, the complement fixation (CF) test was employed. The antigen was a hot saline extract of *B. ovis* strain 63/290. Titers corresponding to ≥ 50 CF test international units/ml were considered positive (Cerri et al., 2000).

Microscopic agglutination test (MAT) was used to detect *Leptospira* antibodies. A panel of 8 serovars was used as live antigen: Icterohaemorrhagiae (strain Bianchi), Canicola (strain Alarik), Pomona (strain Mezzano), Tarassovi (strain Johnson), Grippotyphosa (strain Moscow V), Bratislava (strain Riccio 2), Ballum (strain Castellon 3) and Hardjo (strain Hardjoprajitno). The cultures were grown in Ellinghausen-MacCullough-Johnson-Harris (EMJH—Difco, Detroit, Michigan, USA) at 30 °C for 4–14 days and checked for purity, mobility and agglutination ability. MAT was performed following the procedure previously reported by Cerri et al. (2003). Titers of 1:100 were considered positive; 2-fold serial dilutions were tested to determine the endpoint titer.

Statistical analysis on the distribution of positive sera, farms and serovars related to geographical area was performed using Chi-square test ($P \leq 0.05$ was considered statistically significant).

RESULTS

All sera resulted negative for *B. ovis*.

Seventy-three out of 410 (17.80%) sera were found positive for *Leptospira* antibodies. In particular, 24/170 sera collected in Northwest of Tuscany, 39/187 collected in Southwest and 10/53 collected in Northeast resulted positive (Table 1).

Among the 76 considered farms, 26 (34.21%) presented at least one seropositive animal. Thirteen of them were located in northwest Tuscany, 10 in southwest and 3 in Northeast (Table 1).

Table 1. Tested sera and breeding farms in relation to geographic area.

Provenience	Sera		Breeding Farms	
	Tested	Positive to <i>Leptospira</i> (%)	Tested	Positive to <i>Leptospira</i> (%)
Northwest	170	24 (14.11)	54	13 (24.07)
Southwest	187	39 (20.85)	16	10 (62.50)
Northeast	53	10 (18.86)	6	3 (50.00)
Total	410	73 (17.80)	76	26 (34.21)

Table 2. Number of positive sera and antibody titers detected for each *Leptospira* serovar

Serovars	Titer							Total (%)
	1:100	1:200	1:400	1:800	1:1600	1:3200	1:25600	
Icterohaemorrhagiae	4	1	1	0	0	0	0	6 (1.46)
Pomona	6	3	8	5	4	0	0	26 (6.34)
Grippityphosa	5	3	3	1	1	0	0	13 (3.17)
Tarassovi	0	0	2	0	0	0	0	2 (0.48)
Bratislava	4	2	1	0	0	0	0	7 (1.70)
Hardjo	4	1	2	5	3	1	1	17 (4.14)
Ballum	2	0	0	0	0	0	0	2 (0.48)

Table 3. Distribution of *Leptospira* serovars seropositivity in relation to their provenience

Provenience	Serovars							Total
	Ih	Po	Gr	Ta	Br	Ha	Ba	
Northwest	1	2	12	0	4	5	0	24
Southwest	3	24	1	0	3	6	2	39
Northeast	2	0	0	2	0	6	0	10
TOT	6	26	13	2	7	17	2	73

Ih = *Icterohaemorrhagiae*, Po = *Pomona*, Gr = *Grippityphosa*, Ta = *Tarassovi*, Br = *Bratislava*, Ha = *Hardjo*, Ba = *Ballum*

Twenty-six sera resulted positive for serovar Pomona, 17 for serovar Hardjo, 13 for serovar Grippityphosa, 7 for Bratislava, 6 for Icterohaemorrhagiae and 2 for serovars Tarassovi and Ballum. No sera resulted positive for serovar Canicola. Table 2 reports the MAT titers for each sample.

Table 3 shows the distribution of positive sera in relation to provenience.

DISCUSSION

During routine *Brucella* spp. surveillance program, blood samples from rams were collected in order to extend serological investigation to *B. ovis* and *Leptospira*. Males were selected for the survey because of their involvement in the venereal transmission of these microorganisms (Cerri et al., 1999, Magajevski et al., 2005, Director et al., 2014). Based on our results, *B. ovis* seroprevalence in Tuscany appears to be zero. In accordance with our findings, *B. ovis* infection was not reported in Italy in recent years, even if the disease occurred recently in East Europe and France (Spicić et al., 2009, Petrović et al., 2014, Picard-Hagen et al., 2015).

Concerning *Leptospira* seroprevalence in rams, our results are in accordance with a previous survey carried out on the same geographical area (Cerri et al., 2003). In relation to tested farms, high percentage of positivity was detected. This finding confirms the epidemiological role of the male animals in *Leptospira* spreading and, consequently, their primary function as “detectors” of the presence of infection in the farm. Concerning *Leptospira* serovars, Pomona was the most represented (6.34% positive subjects). The high percentage detected for this serovar, which is unusual for sheep, could be probably explained by the presence of wild boars in the areas where the sampling was conducted, which represent maintenance hosts for Pomona (Żmudzki et al., 2016). The second most detected serovar was Hardjo (4.14%). These results were expected because sheep represent the second maintenance host for this serovar (Farina et al., 1996). A high percentage of seropositive animals (3.17%) for serovar Grippityphosa was also detected. This serovar was reported all over Europe in the past years, in both wild and domestic animals. Also, infection in sheep with Grippityphosa serovar could be related to the breeding farm management, as suggested

by other Authors (Andreoli et al., 2014, Ayral et al., 2014, de Carvalho et al., 2014, Źmudzki et al., 2016). In particular, the presence of this serovar in Tuscany could be related to hares' importation from eastern Europe for repopulation and hunting purposes (Trembl et al., 2007, De Massis et al., 2012). In this study, antibody titers of 1:100 were considered as threshold; titers of 1:100 or 1:200 may correspond to the early stage of the infection, to a previous infection or to vaccinal antibodies, whereas, titers of 1:400 could be representative of infection in endemic areas (Picardeau, 2013). The highest antibody titers were obtained for serovars Hardjo, with values ranging from 1:100 to 1:25600, and for serovars Grippotyphosa and Pomona, with values ranging from 1:100 to 1:1600. These results suggest some probable leptospirosis outbreaks. Concerning other serovars, the sporadic and low antibody titers detected show the spreading of these serovars as well. Grippotyphosa serovar was mainly represented in northwest Tuscany and

Pomona serovar in the southwest, while for other serovars no differences were observed in relation to the geographical distribution.

CONCLUSION

In conclusion, *B. ovis* infection seems to be absent in the investigated area, however, in light of recent spreading of this disease in some European countries, continuous surveillance would be useful. Our results highlight the occurrence and spreading of *Leptospira* in sheep in Tuscany. Moreover, infection by serovar Hardjo in sheep is probably underestimated and spreading of serovar Grippotyphosa and Pomona could represent a potential hazard for other animals, such as dogs and cattle. Further serological and bacteriological investigations should be carried out on a higher number of samples from wild and domestic animals, in order to determine more accurately the prevalence of leptospirosis in Tuscany and clarify its potential health risk. ■

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