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Original Article

High frequency of seropositivity of *Leptospira* in cattle in North Tunisia

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Abstract

The authors report a survey carried out in a cattle farm between January and April 2009 in Mateur region (Northern Tunisia). Seroprevalence by Microscopic Agglutination Test (MAT) was estimated to 81.4 ± 6% and 35 ± 2% in cows and calves, respectively. Seropositivity to more than one serovar was noticed in 91% (81/89) of infected animals. The examination of the distribution pattern of *Leptospira* serovars involved in this outbreak indicates that serovar Pomona was the predominant one (75.3%), followed by Autumnalis (59.5%), Bim (58.4%) and Munchen (55%). High titres (between 400 and 6400) were found in 68.7% of the tested animals and were correlated with clinical onset of leptospirosis. Leptospirosis is an underestimated pathogen in Tunisia; further investigations are needed to study the epidemiology both in man and animals and to implement effective control measures.

Keywords: Cattle, Cross-reactivity, *Leptospira*, Microscopic Agglutination Test, Pomona, Tunisia.

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Introduction

Leptospirosis (*Leptospira* spp. infection) is a cosmopolitan spirochetal disease of humans and animal occurring especially in tropical and subtropical regions (Desvars *et al.* 2011). Despite the progress in molecular taxonomy, the subdivision of the genus in to serogroups and serovars remains widely used (Picardeau 2013). Each serovar has its own distribution area and its own host maintenance species. The prevalence of infection of rodents, which are the main reservoirs of leptospires, varies between 10.5 and 90.5% in Japan (Koizumi *et al.* 2008) and USA (Vinetz *et al.* 1996) respectively.

In cattle, leptospirosis induces decreased milk yield and reproductive failure due to infertility, abortions and stillbirths (Thiermann, 1984). The most important serovars, affecting cattle is L. Hardjo followed by Pomona (Bharti *et al.* 2003; Grooms 2006; Salgado *et al.* 2014). In Morocco, a survey carried out in North West regions reveals that 15% of cattle were seropositive to serovars Ballum, Sejroe and Australis (Benkirane *et al.* 2014). In Egypt the seroprevalence of bovine leptospirosis is estimated to be between 37.6 and 40% (Horton *et al.* 2014).

In Tunisia, the only study concerning bovine leptospirosis was in the Kef region (Northwest Tunisia) and was undertaken more than half a century ago by Durand & Loquerie (1960). The authors reported two clinical cases in a herd of 40 cattle. Fever, icterus and redwater were the main clinical signs exhibited by affected animals. Laboratory examination using culture and guinea-pigs inoculation confirmed *Leptospira* spp. infection.

In sheep, the seroprevalence in Central Tunisia has been estimated to be 25% (Khamassi Khbou *et al.* 2010). Serovar Copenhageni was present in 98% of sheep. As far as the authors know, there are no recent studies of the prevalence of leptospirosis in both humans and domestic animals in Algeria, Tunisia and Libya.

The Microscopic Agglutination Test (MAT) is the reference serological test for *Leptospira* diagnosis [Office International des Epizooties (OIE) 2014]. To be most relevant, the MAT should be performed using local live *Leptospira* strains; because the results are interfered with by occurrence of cross-reactivity between serovars. However, according to Levett (2003) and Blanco *et al.* (2016) the highest titres in MAT correlate to the cultured serogroup, making this test a good indicator of the infecting serovars.

In this study we performed a cross-sectional serological survey in a cattle farm in Northern Tunisia where an outbreak of leptospirosis occurred in 2009. The authors examine distribution pattern of *Leptospira* serovars and the multiple serovars cross-reactions.

Materials and methods

Animals

This study was carried out in an intensive dairy farm located in Mateur, Governorate of Bizerte (Northern Tunisia). Mateur (longitude 9.6 E; latitude 37.0 N, altitude 41 m asl) is a sub-humid region with an annual mean rainfall of 539 mm. The mean temperature in winter and summer are 6 and 33°C, respectively.

The farm consisted of approximately 1000 cattle heads lodging in 5 units of about 200 animals; the distance between the units is 60 to 350 metres. The cows are lodged in free-stalls while calves lived in individual pens.

The occurrence of fever, icterus, redwater and mortality in calves and cows led to the suspicion of leptospirosis in this herd. In addition, recurrent milk yield decrease and reproductive problems were reported in this farm, i.e. low fertility, abortions and stillbirths. A representative number (10%) of the cattle population was randomly sampled from 4 units. Sera samples were collected in sterile vacutainer tubes from 102 cows (aged between 2 and 8 years) and 17 calves (aged between 2 and 12 months).

Serology

The sera were tested for *Leptospira* spp. antibodies using the standard microscopic agglutination test (MAT) at 1/100 cut-off value (Faine *et al.* 1999). A titre equal or above 1/400 was considered to be high and correlated with acute clinical leptospirosis (André-Fontaine 2016). Briefly, panels of fourteen live reference strains of leptospires (Laboratory of Leptospires Diagnosis, National Veterinary School of Lyon, France) in exponential stage of growth were used as serogroups and serovars standards. Sera samples were serially diluted in phosphate-buffered saline (PBS) at 2-fold dilutions, from 1:50 to 1:6400 and mixed with the same volume of leptospires suspension. Two hours after incubation at 30°C, the plates were read under a dark-field microscope at 250× magnification. The MAT titre corresponds to the reciprocal of the highest dilution of the serum in which more than 50% of the leptospires agglutinated. Positive and negative standard sera and one antigen control were used in each run.

Nine tested serogroups consists of more than one serovars, only Pomona is single serovar in its serogroup.

Statistical analysis

Chi square test was performed with EpiInfo 2000 (CDC, Atlanta, USA) at 5% threshold. Principal components analysis (PCA) was performed with XLstat software for windows (Microsoft ©, WA, USA).

Results

Seroprevalences

The overall seroprevalence of anti-*Leptospira* spp. antibodies in cattle was 74.8 ± 7.8%. The seroprevalences were 81.4% (83/102) and 35% (6/17) in cows and calves, respectively ($P < 0.0001$) (Table 1).

Antibody titres

Positive sera titres ranged between 1/100 and 1/6400 with a median of 1/800 (Table 2; Fig. 1). More than half of the sera (68.7%; 332/483) reacted to at least one serovar at high titres (≥1/400). Three animals developed the highest titres (1/6400) for serovar Bratislava and one for serovar Munchen. High antibody

Table 1. Seroprevalences of *Leptospira*-antibodies in cattle

Cattle unit	Cows Total number	Positive/tested	Calves Total number	Positive/tested	Overall Total number	Positive/tested (%± S.E.)
1	198	24/32	39	0/2	237	24/34 (70.6 ± 15.3)
2	192	14/14	32	3/5	224	17/19 (89.5 ± 13.8)
3	383	14/25	32	0/6	415	14/31 (45.2 ± 17.5)
4	201	31/31	38	3/4	239	34/35 (97.1 ± 5.5)
Overall	974	83/102	141	6/17	1115	89/119 (74.8 ± 7.8)

S.E.: Standard Error.

Table 2. Number of animals reacting to 14 *Leptospira* serovars in Microscopic Agglutination Test at different titres

Serogroup	Serovar	Antibodies titres 1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	Total (%)
Pomona (POM)	Pomona (POM)	0	6	5	26	24	6	0	67 (13.9)
Autumnalis (AUT)	Autumnalis (AUT)	4	7	6	16	16	4	0	53 (11)
	Bim (BIM)	3	7	14	14	9	5	0	52 (10.8)
Icterohaemor-raghiae (ICT)	Copenhageni (COP)	0	4	7	22	0	0	0	33 (6.8)
	Icterohaemorraghiae (ICT)	8	8	10	4	0	0	0	30 (6.2)
Australis(AUS)	Australis (AUS)	3	5	9	5	0	0	0	22 (4.6)
	Munchen (MUN)	1	17	12	18	0	0	1	49 (10.1)
	Bratislava (BRA)	9	17	11	2	2	2	3	46 (9.5)
Sejroe (SEJ)	Hardjo (HAR)	6	13	17	4	2	0	0	42 (8.7)
	Sejroe (SEJ)	7	11	18	6	1	0	0	43 (8.9)
	Wolffii (WOLF)	1	12	19	5	2	2	0	41 (8.5)
	Saxkoebing (SAX)	0	0	1	0	0	0	0	1 (0.2)
Panama (PAN)	Panama (PAN)	0	1	1	0	0	0	0	2 (0.4)
	Mangus (MAN)	0	1	0	1	0	0	0	2 (0.4)
Total (%)		42 (8.7)	109 (22.6)	130 (26.9)	123 (25.5)	56 (11.6)	19 (3.9)	4 (0.8)	**483**

In bolded characters: The total number of positive reactions to all tested serovars

titres to serovar Pomona were detected in 91% (61/67) of the tested animals (Table 2; Fig. 1). As sera of infected animals reacted to more than one serovar, the number of total seropositive animals (n = 89) was different from number of positive reactions to all tested serovars (n = 483).

The number of animals that developed the higher titre expressed against one or more serovars, is shown in the Figures 2 and 3. Out of 67 positive reactions to serovar(s) Pomona, 53 expressed the highest titre to this serovar alone or in addition to other serovars.

Serovars

Animals reacted to all tested 14 serovars in the farm. Regardless of the highest titre cut-off, the most prevalent serovars were Pomona (75.3%), followed by Autumnalis (59.5%), Bim (58.4%) and Munchen (55%) ($P < 0.05$) (Fig. 2). The serovar Pomona was the most frequent in units 1, 2 and 4. In unit 3, out of the 14 seropositive sera tested, 13 reacted to one or more serovars from the serogroup Sejroe and one sera was positive to Pomona.

Only eight animals reacted to a single serovar (Pomona), cross-reactivity between at least two serovars were detected in 91% of positive animals (81/89), among them seven reacted to 11 serovars at the same time (Fig. 3). Among positive animals to serogroup Sejroe, 37/48 showed cross-reactivity to at least three serovars (Sejroe, Hardjo and Wolffii (n = 36); Sejroe, Wolffii and Saxkoebing (n = 1)). As expected, more than half of the animals (25/49;

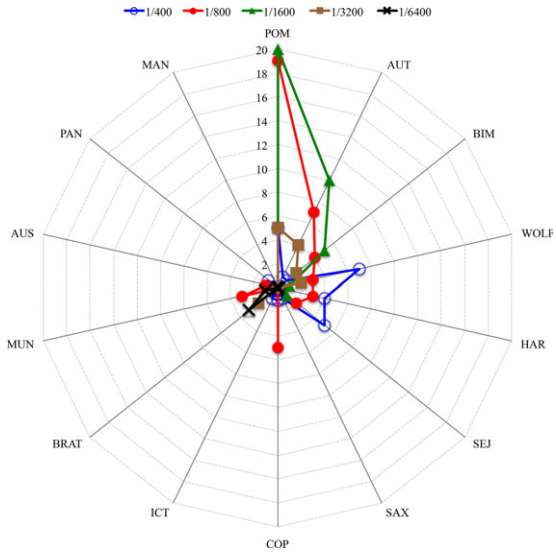

Fig. 1. Number of animals reacting to different *Leptospira* serovars with the highest titres.

51%) reacted to the serogroup Australis; they expressed cross-reactivity between two serovars (Munchen and Bratislava) and 21/49 (42.85%) to three serovars (Munchen, Bratislava and Australis). Most of the seropositive animals to serogroup Icterohaemorrhagiae (30/33; 90.9%) expressed cross-reactivity to the only two serovars (Icterohaemorrhagiae and Copenhageni). Roughly all animals positive to serogroup Autumnalis, reacted to both serovar Bim and serovar Autumnalis (52/53). The same observation was made for Panama and Mangus (2/2) (Fig. 4).

The Principal component analysis (PCA) showed high correlation values mainly between serovars belonging to the same serogroups (Fig. 5; Table 3). Curiously, animals developed cross-reactivity against Icterohaemorrhagiae, Australis and Autumnalis serogroups, and with less extent between Pomona and Icterohaemorrhagiae, Australis and Autumnalis serogroups. Sejroe did not cross-react with any of other serovar.

Discussion

This study showed a high seroprevalence of *Leptospira* spp. antibodies (74.8 ± 7.8%) and confirmed a leptospirosis outbreak in the studied farm. High antibody titres (≥1/400) were reported in 68.7% of seropositive animals indicating an active infection. In North Africa, few data are available about leptospirosis in both humans and animals. In Centre of Tunisia, we showed lower seroprevalence (25%) in sheep with no leptospirosis clinical cases (Khamassi Khbou *et al.* 2010).

In Egypt, the seroprevalence with MAT varied between 37.6 and 44.4% (Felt *et al.* 2011; Samir *et al.* 2015). The seroprevalences with MAT in Northern Morocco and Middle Atlas were 5 (5/99) and 1% (1/98) in goats and sheep with abortion history respectively (Benkirane *et al.* 2015).

According to the highest titre expressed by sera, Pomona was the predominant serovar (79.1%; 53/67). This serovar(s), was reported in other countries in cattle, by MAT in Iran (13/29) (Khalili *et al.*

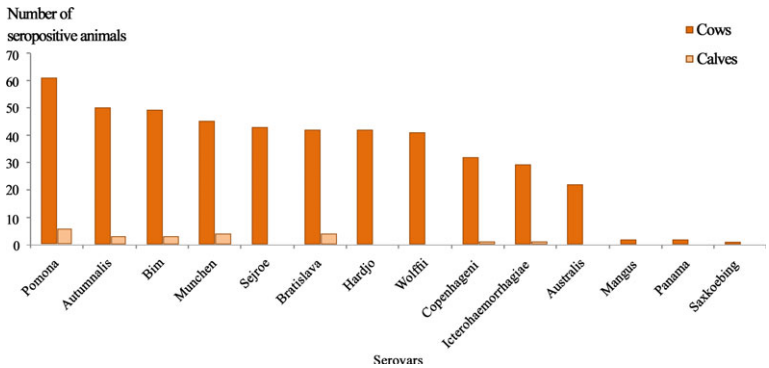

Fig. 2. Number of seropositive animals reacting to against the 14 tested *Leptospira* spp. serovars.

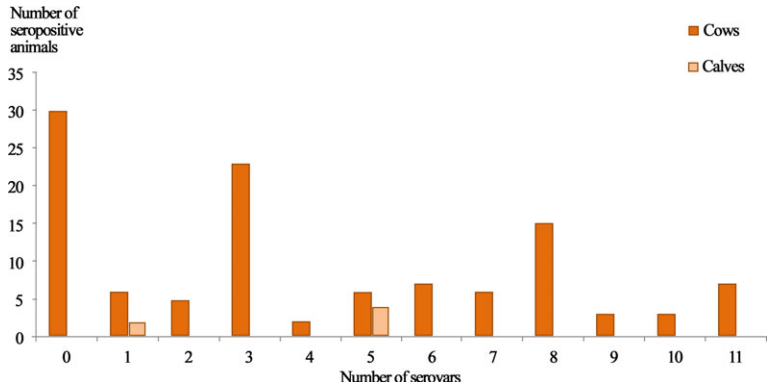

Fig. 3. Number of animals showing seropositivity to different *Leptospira* serovars.

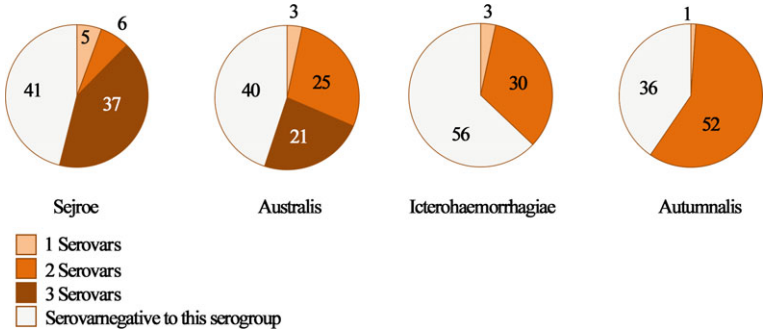

Fig. 4. Frequency of serovars' associations belonging to the same *Leptospira* serogroup.

2014); or by culture and PCR in Egypt (5/7) (Hatem *et al.* 2014). Serovar Pomona causes leptospirosis in cattle (Bharti *et al.* 2003) and is involved in foetal losses and abortion among non-vaccinated cows (Sanhueza *et al.* 2013). As in Tunisia there are no pigs, the main reservoir of serovar Pomona (Bharti *et al.* 2003), rodents may play this role but further studies are needed to confirm this role.

In contrast, Hardjo (Sejroe serogroup) is considered as the most prevalent serovar in cattle in England, Columbia, Chile and Brazil (Ellis *et al.* 1981; Hernández-Rodríguez *et al.* 2011; Lilenbaum & Martin 2014; Salgado *et al.* 2014). In France between 1988 and 2007, the most frequent serovar in cattle was Serjoe (34%) (André-Fontaine 2016). Similar trend was observed in the unit 3 of the present survey (13/14). As in this unit, the cross-reactivity with other serovars was low (Pomona, Panama and Man,

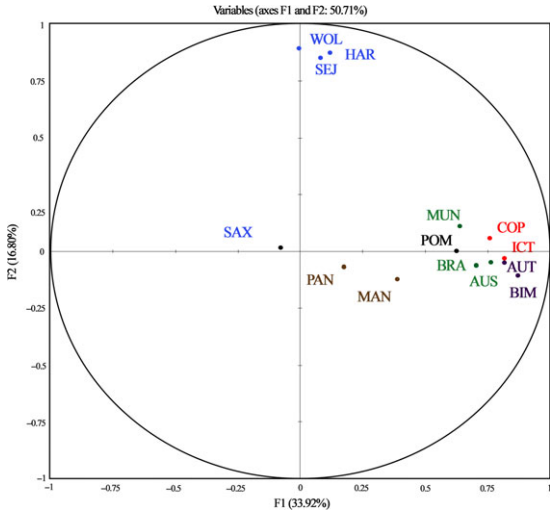

Fig. 5. Principal component analysis of *Leptospira* spp. serovars associations. **Bleu**, serovars of Sejroe serogroup; **Green**, serovars of Australis serogroup; **Red**, serovars of Icterohaemorrhagiae serogroup; **Purple**, serovars of Autumnalis serogroup; **Brown**, serovars of Panama serogroup .

Table 3. Correlation matrix of the Principal components analysis for different serovars

		Serogroup													
		ICT		AUS			AUT		SEJ				PAN		POM
Serogroup	Serovar	COP	ICT	AUS	MUN	BRA	AUT	BIM	HAR	SEJ	WOL	SAX	PAN	MAN	POM
ICT	**COP**	1	**0.684**	**0.564**	**0.452**	**0.358**	**0.563**	**0.546**	0.123	0.105	0.010	−0.053	0.035	0.166	**0.450**
	ICT		1	**0.692**	**0.409**	**0.503**	**0.636**	**0.663**	0.026	0.080	−0.038	−0.042	0.037	0.150	**0.365**
AUS	**AUS**			1	**0.616**	**0.503**	**0.514**	**0.641**	0.048	0.001	−0.020	−0.037	0.127	**0.336**	**0.334**
	MUN				1	**0.321**	**0.318**	**0.574**	0.137	0.089	0.080	−0.035	0.004	0.070	**0.305**
	BRA					1	**0.358**	**0.630**	0.080	0.005	−0.014	−0.028	0.207	**0.495**	**0.442**
AUT	**AUT**						1	**0.695**	0.036	0.051	−0.064	−0.057	0.011	0.121	**0.592**
	BIM							1	0.005	−0.019	−0.062	−0.051	0.109	**0.324**	**0.478**
	HAR								1	**0.630**	**0.702**	−0.047	−0.033	−0.049	0.128
SEJ	**SEJ**									1	**0.665**	0.022	0.030	0.027	0.043
	WOL										1	0.038	0.017	−0.028	−0.068
	SAX											1	−0.011	−0.010	−0.075
PAN	**PAN**												1	**0.646**	−0.022
	MAN													1	0.079
POM	**POM**														1

In bolded characters: statistically significant values. The intensity of the green colour is proportional to the r correction value.

one of each) and high titres (>3200) were expressed against serovar Wolffii, we can argue that in this unit, the animals were infected by a serovar belonging to the Sejroe serogroup.

Roughly all the tested animals (81/89) were seropositive to more than one serovar. Seropositivity to three serovars was the most frequent (23/81), and surprisingly, seven animals were positive to 11 serovars. Allan *et al.* (2015) reported that cattle are carriers of the widest range of *Leptospira* serogroups, but in our study, we cannot argue that positive animals that reacted to multiple serovars were co-infected, as co-infection is only confirmed by *Leptospira* culture or PCR. The seropositivity to several serovars is likely to be due to cross-reactivity, which is more intense between serovars belonging to the same serogroup (Faine *et al.* 1999). Based on highest titres, Pomona remains the predominant serovar, even in animals that showed high titres against multiple serovars. The combination of Pomona and Autumnalis was the most frequent among animals that strongly reacted to several serovars.

Antibodies are produced against species serovar-specific and serogroup-specific antigens (World Health Organization (WHO) 2003; Seenichamy *et al.* 2014). Although, cross-reactivity to multiple serovars is considered as a limit of MAT (Suepaul *et al.*, 2011), when it is associated to high titres, it could indicate the pattern of the infected serogroups, as animals develop an intense immunological response in MAT to the first causative *Leptospira* serovar with higher titres than the others (Chappel *et al.* 2004; Ayral *et al.* 2014; André-Fontaine 2016; Blanco *et al.* 2016). If just the highest titre values are considered, it can be argued that Pomona was the predominant infecting serovar in three cattle units (1; 2 and 4), but in the cattle unit 3, serovar Sejroe was the most prevalent (13/14).

This study showed the presence of high seroprevalence with high titres against *Leptospira* serovar Pomona. The prevalence reported herein might be underestimated because we did not use local *Leptospira* serovars from Tunisia for MAT, and this could induces a decrease in MAT sensitivity (Office International des Epizooties (OIE) 2014; Mgode *et al.* 2015).

Nationwide surveys targeting on one hand different animal species in different Tunisian geographical regions should be carried out. At the same time, a seroepidemiological survey should be carried out in human Tunisian population to establish a list of the serovars that are mostly involved in human cases. These surveys will provide both human and animal health decision makers and practitioners with reliable and comprehensive epidemiological data to control this neglected disease.

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Conflict of interest

There are no potential conflicts of interest.

Contribution

M. Khamassi Khbou: participated in sample collection, data analyses and wrote the paper. K. Haouala: participated in sample collection and coordinated the whole survey in field. M. Benzarti: designed the experiment.

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