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Seroprevalence of anti-*Mycobacterium avium* subsp. *paratuberculosis* antibodies in female sheep in Tunisia

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Abstract

Paratuberculosis (Ptb) is a widespread chronic infection caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) that affects both domestic and wild ruminants. Most of the studies focused on cattle while, the prevalence of Ptb in sheep in different regions of the world is not well investigated. This study aimed to address this gap of knowledge by screening adult female sheep for paratuberculosis antibodies in different geographical regions of Tunisia. A total number of 338 female sheep from 15 small to middle-sized, extensively managed sheep farms in six regions across Tunisia were sampled. Animals were clinically examined before blood sampling. Sera were tested for the presence of anti-*Mycobacterium avium* subsp. *paratuberculosis* antibodies using a commercial ELISA kit. Six farms out of 15 comprised at least one seropositive animal and 11 female sheep out of 338 tested animals (3.25%; 95% CI = [1.83–5.73]) were seropositive to *M. avium* subsp. *paratuberculosis*. The seroprevalence was significantly lower in 5-year-old females (p = .04) and animals that do not graze (p = .02). Due to its huge economic and social impacts, paratuberculosis represents a health problem in Tunisia and several other countries Further investigations are needed to rank sheep Ptb in Tunisia among other diseases and to assess the main risk factors using a larger nation-wide survey.

KEYWORDS
chronic, ELISA, *Mycobacterium* subsp. *paratuberculosis*, paratuberculosis, sheep, Tunisia

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1 | INTRODUCTION

Paratuberculosis (Ptb) is a widespread chronic infection caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) and affects both wild and domestic ruminants (Harris & Barletta, 2001). It was recently reported in dromedaries in Egypt and Saudi Arabia (Salem, El-deeb, Zaghawa, Housawi, & Alluwaimi, 2019; Salem, El-Sayed, Fayed, & Abo-El-Hassan, 2012). *Mycobacterium avium* subsp. *paratuberculosis* is incriminated to play a role in Crohn's disease in humans, but this fact is still not confirmed (Pierce, 2018). Young animals are infected mainly through faecal-oral way by ingestion of contaminated milk or forage at older ages (Rathnaiah et al., 2017). Paratuberculosis in adult ruminants induces chronic enteritis that is manifested by intermittent diarrhoea and weight loss (Lashner and Brzezinski, 2010). Small proportion of cattle would develop chronic severe water diarrheal and emaciation; whereas in sheep and goats, the infection is more discrete and insidious (Windsor & Whittington, 2010). In small ruminants, Ptb is restricted to occasional emission of pasty faeces in the advanced stages of the disease with no hyporexia. Thus, small ruminants seem to act as reservoir of bacteria for cattle and even for non-ruminant wildlife species like rodents, hares and foxes (Florou et al., 2008). This role is fostered by the high resistance of *Map* in the environment that was estimated to up 250 days in water, faeces and slurry (Harris & Barletta, 2001).

Paratuberculosis in sheep and goats was diagnosed in many countries including those of Mediterranean sea, Europe, Southern hemisphere (Australia and New Zealand) and Canada (Windsor, 2015). The seroprevalence ranged between 6.29% (129/2086) and 48.3% (192/397) in Italy and Ontario, respectively (Attili et al., 2011; Bauman et al., 2016).

In Tunisia, sheep population was estimated around 3,736,820 females distributed at 38%, 43% and 19% in the North, Centre and South, respectively (Ministry of agricultural, 2018). The seroprevalence of Ptb in sheep was estimated to 1.09% in Siliana district (Zribi, 2010), whereas in goats, it ranged between 0.15% and 1.31% in Tataouine and Siliana districts, respectively (Hdia, 2008; Zribi, 2010). In cattle, Habchi (2006) screened for the prevalence of Ptb in state-owned cattle farms in various regions of the country and found that 5.4% (n = 1,032) of animals were seropositive. In a study comparing in vitro culture, Ziehl-Neelsen staining and ELISA to investigate Ptb infection in a cattle farm with chronic diarrhoea and weight loss in the North West of Tunisia and out of 65 tested animals, 30 (46%), 36 (55%) and 8 (12%) were positive to ELISA, Ziehl-Neelsen staining and in vitro culture, respectively (Chariet, 2004). Among the 30 seropositive cattle, 10 were developing diarrhoea. As Ptb is well established in Tunisia particularly in cattle, it was included since 2009 in the list of Regulated Animal Diseases (a list of animal diseases requesting special recommendations) (JORT, 2009). Despite this classification, the prevalence of Ptb remains unknown in several Tunisian regions, especially in small ruminants together with an increased incidence for Crohn's disease within the population (Ouakaa-Kchaou et al., 2013). This study aims to address this gap in knowledge by screening adult female sheep for paratuberculosis antibodies in different geographical regions of the country.

2 | MATERIALS AND METHODS

Sera were collected from female sheep during October 2018, for survey on ticks and tick-borne pathogens. The sampled animals were

TABLE 1 Characteristics of surveyed farms

Location (District)	Farms (number of tested animals per farm)	Bioclimatic stratus	Latitude	Longitude	Mean altitude (m)	Total number of female sheep in the district*
Fernana (Jendouba)	1 (33)	Humid	36°42'20.54"	8°48'46.5"	500	137,110
Mornaguia (Manouba)	2 (42)	Sub-humid	36°37'20.84"	9°56'55.63''	122	45,000
	3 (13)		36°39'38.58''	9°57'33.53''	121	
	4 (6)		36°46'30.86''	9°57'27.66''	57	
Saouef (Zaghouan)	5 (15)	Semi-arid	36°14'52"	10°9'7"	205	179,900
	6 (33)		36°15'27"	10°8'49"	184	
	7 (17)		36°13'41.54"	10°10'47.17"	151	
Sbeïtla (Kasserine)	8 (21)	Arid high steppes	35°17'44"	9°14'35.93"	470	309,000
	9 (35)		35°17'44"	9°14'35.93"	470	
	10 (19)		35°14'28.36"	9°5'28.66"	561	
Bir Ali (Sfax)	11 (11)	Arid low steppes	34°38'75.9"	10°4'46.4"	117	196,100
	12 (47)		34°46'19.4"	10°2'30.3"	203	
Bir Lahmar (Tataouine)	13 (16)	Saharian	33°7'4.22"	10°33'42.13"	142	128,680
	14 (23)		33°7'31"	10°33'54"	149	
	15 (7)		10°35'14.39"	10°35'14.39"	131	

*(Ministère de l'agriculture, 2018).

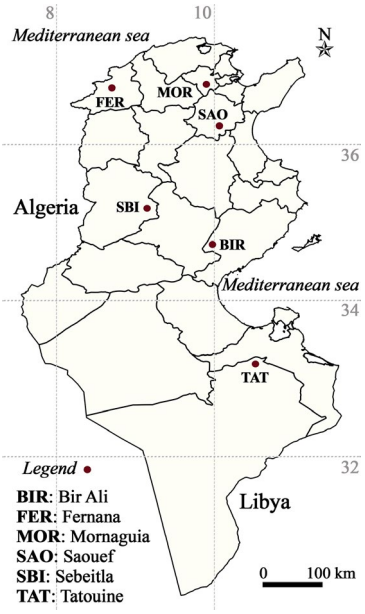

FIGURE 1 Map of Tunisia indicating the sheep farms' locations (red dots)

belonging to 15 small to middle-sized (10 to 50 ewes par herd) and extensively-managed sheep farms in six regions across Tunisia and meant to represent different natural environments along the main sheep production areas in the country. In each herd, all female sheep aged more than 1-year old, were selected with corresponded to a total number of 338 animals (Table 1). The sampling sites are Fernana, Mornaguia, Saouef, Bir Ali, Sebeitla and Tataouine (Table 1; Figure 1). Sheep graze all around the year on natural rangelands and cereal stubbles in summer particularly in the case of the north-located farms. Farmers supplement their sheep with concentrate especially in winter. Water is provided either from public or natural resources and is more available in Northern Tunisia (Ibidhi, Frija, Jaouad, & Hichem, 2018). Sheep are reared in mixed flocks with goats and with cows in the case of five farms. Spring is the main mating season and most births are spread during autumn and early winter (September–February).

One hundred and five (31.06%), 121 (35.8%) and 106 (31.36%) were from the breed Brabarine (fat-tailed breed), Queue Fine de l'Ouest (fine-tailed breed) and cross-breeds, respectively. All the animals were clinically examined for their temperature (fever threshold value: 39.5°C), the eye mucosa status (congested or normal), the macroscopic aspect of their faeces (pasty or solid) and their body score was estimated from bad (score 1) to excellent (score 4).

Five millilitres of blood in sterile dry tubes were collected from the jugular vein of each animal using a vacutainer. Sera were collected in Eppendorf tubes and were stored at −20°C until used.

The sera were tested for the presence of anti-*Mycobacterium avium* subsp. *paratuberculosis* antibodies using a commercial ELISA kit (IDEXX Paratuberculosis Screening® kit, IDEXX, Montpellier, France) according to the manufacturer's instructions.

The ELISA plates were read with a spectrophotometer (Multiscan^{TM}FC, ThermoFisher Scientific, Waltham, MA) at 450 nm length wave to determine the optical density (OD) of each serum. The results were expressed as ratio of OD sample/OD-positive control (OD_S/OD_{PC}), corrected for the negative control (OD_{NC}). Negative and positive control sera provided with the kit were added in each plate. The ELISA plates were validated if the mean of optical density for two positive control sera $OD_{PCx} > 0.3$ and $OD_{PCx}/OD_{NCx} > 3$. A serum sample was considered positive if $O_{DS}/OD_{PC} > 0.45$ and negative if $OD_S/OD_{PC} \leq 0.45$.

Data were analysed using SPSS version 21 software (IBM, USA). Chi square or Fisher exact test were used at 5% threshold value to check the relation between different variables and animal's serological status. The 95% confidence intervals for proportions were estimated (Schwartz, 1993).

3 | RESULTS

Eleven female sheep out of 338 (3.25%; 95% CI = [1.83–5.73]) were found seropositive to *M. avium* subsp. *Paratuberculosis* and six farms out of 15 comprised at least one seropositive animal. The highest seroprevalence was recorded in farms in Saouef district (9.2%, 95% CI = [4.3–18.7]) and the lowest in Sebeitla district (1.3%, 95%CI=[0.2–7.1]) but the difference was not statistically different (p = .08). No seropositive female sheep were detected in both Fernana and Tataouine districts (Table 2).

Four risk factors were tested for their association with Ptb seropositivity: age, grazing, presence of cattle and goats in the farm (Table 3). The seroprevalence was significantly lower in 5-year old and older females (p = .04) and animals that do graze (p = .02).

4 | DISCUSSION

Despite the financial impact of paratuberculosis that was estimated in sheep to $90 per clinical case, few studies focused on sheep Ptb (Menzies & Jansen, 2011). The low venal value of sheep associated to low disease prevalence of Ptb in most sheep flocks probably made sheep Ptb a neglected health issue in several countries (Windsor, 2015).

As sera were collected following a convenient sampling method, the seroprevalence of Ptb in only the selected herds in six Tunisian regions was estimated to 3.25%. It is a relatively low rate and is consistent with previous data reported in sheep in Tunisia by Zribi (2010) (1.09 ± 1.47%). Under a similar Mediterranean environment, the seroprevalence of Ptb in Italy was estimated in ewes to 6.29% (129/2086) (Attili et al., 2011), whereas Benazzi, Berrada, and Schliesser (1995) reported a low prevalence in Morocco (1%; 10/1000). In Saudi Arabia, Mahmoud, Haroun, Elfaki, and Abbas (2002) reported one case in sheep in a slaughterhouse, with gross macroscopic intestinal lesions and pigmentation on the liver that was positive to Ziehl-Nielsen staining and culture. The low prevalence is due to less receptivity of sheep to Ptb compared to cattle (Windsor, 2015) and could explain why the epidemiology of Ptb is

TABLE 2 Seroprevalence of paratuberculosis in female sheep according to location, breed, body score condition and faeces aspect

Variable	Positive/examined (%)	[95% CI]	*p*
Farm (*n* = 15)			.08
Fernana	*0/33*[a]		
1	0/33		
Mornaguia	*2/61 (3)*[a]	*[0.9–11.2]*	
2	2/42 (4.7)	[1.3–15.8]	
3	0/13		
4	0/6		
Saouef	*6/65 (9)*[a]	*[4.3–18.7]*	
5	1/15 (6)	[1.2–29.8]	
6	1/33 (3)	[0.5–15.3]	
7	4/17 (23)	[9.5–47.7]	
Sbeïtla	*1/75 (1.3)*[a]	*[0.2–7.2]*	
8	0/21		
9	0/35		
10	1/19 (5)	[0.9–24.6]	
Bir Ali	*2/58 (3)*[a]	*[0.9–11.7]*	
11	0/11		
12	2/47 (4)	[1–14]	
Tataouine	*0/46*[a]		
13	0/16		
14	0/23		
15	0/7		
Sheep breed (*n* = 332)			.5
Barbarine	5/105 (4.7)	[2–10.6]	
Queue fine de l'ouest	2/106 (1.8)	[0.52–6.6]	
Cross-breed	4/121 (3.3)	[1.3–8.2]	
Body score condition (*n* = 338)			.1
1 (Bad)	0/5 (0)	N.A.	
2 (Medium)	0/82 (0)	N.A.	
3 (Good)	6/174 (3.4)	[1.6–7.3]	
4 (Excellent)	5/77 (6.5)	[2.8–14.3]	
Faeces aspect (*n* = 314)			.4
Diarrhoea	0/3 (0)	N.A.	
Pasty	0/49 (0)	N.A.	
Solid	9/262 (3.4)	[1.8–6.4]	

Abbreviations: N.A., not applicable.

[a]Italic values correspond to subtotals.

TABLE 3 Risk factors for Paratuberculosis seropositivity among sampled animals

Factor	Positive/examined (%)	*p*	OR [95%CI]
Age (years)			
[1–3]	5/67 (7.4)	.04*	N.A.
[3–5]	4/103 (3.8)		
>5	2/157 (1.7)		
Grazing			
Yes	3/212 (1.4)	.02*	0.2 [0.05–0.78]
No	8/115 (6.9)		
Presence of cattle			
Yes	10/194 (5.1)	.06	6.85 [0.86–54.19]
No	1/133 (0.7)		
Presence of goats			
Yes	10/249 (4)	.46	3.13 [0.39–24.85]
No	1/78 (1.3)		

Note: N.A. not applicable.

*Statistically significant *p* values.

not as well characterized in sheep in many countries compared to cattle (Munjal, Boehmer, Beyerbach, Strutzberg-Minder, & Homuth, 2004).

Despite culture is considered as 'Gold standard' test for the confirmation of Ptb, we used ELISA as it is easy to perform, the result is quickly available and it is cheaper than bacterial culture (Hemida & Kihal, 2015). In small ruminants, the sensitivity and specificity of ELISA are 16%–100% and 79%–100%, respectively (Nielsen & Toft, 2008). However, as the antibody response occurs late after primo-infection, the sensitivity of ELISA is higher in clinically infected than sub-clinically infected animals (Milner, Lepper, Symonds, & Gruner, 1987; Milner et al., 1990; Munjal et al., 2004). This means that in our study, the obtained seroprevalence might be underestimated. Indeed, antibodies are detected late during the infection onset and some infected sheep remain seronegatives, even when clinical signs are expressed. Several authors argued that in order to increase sensitivity of detection of *Map* in sub-clinically tested animals, a parallel testing combining serology and PCR is recommended (Munjal et al., 2004; Muskens, Bakker, De, & Van, 2001).

Paratuberculosis is well established in cattle in Tunisia, the seroprevalence was estimated between 5.4 (56/1032) and 21% (36/170) in intensive and extensive farms, respectively (Habchi, 2006; Touihri, 2008). As in extensive herds in Tunisia, sheep are mixed with cattle, the transmission of *Map* between both species is rather to occur frequently.

The absence of Ptb infection in Tataouine district could be explained by the saharian climate, as the high temperature in combination with very low hygrometry may lead to a significant reduction of the *Map* pathogen in the environment. In Fernena, the naught seroprevalence could be biased by the sample; the tested number of animals was low in one single farm.

In Saouef, the seroprevalence was the highest. Animals in Saouef region do not graze, and stay in pens along the day, hence reinforcing lack of hygiene and stimulating transmission of *Map*.

Because all the seropositive animals were clinically normal, they showed no clinical signs of Ptb (diarrhoea, emaciation). These animals could play an important epidemiological role as reservoirs of bacteria especially if they are in contact with cattle.

The seropositivity was higher in younger group than it was in older one and this trend was also reported in cattle (Windsor & Whittington, 2010). Hailat et al., (2010) reported that half of apparently healthy Awassi sheep (n = 202), aged between 8 and 24 months, were Ptb positive at histopathology. Indeed, the 'open gut' of ruminant at birth facilitates the infection by *Map* through the digestive mucosal barrier (Sweeney, Whitlock, Hamir, Rosenberger, & Herr, 1992). It was shown that 14.4% (12/83) of calf milk replacer samples obtained from milk collected in dairy cattle farms in USA, were *Map* culture positive (Grant et al., 2017), which enhances the oral transmission of bacterium to new born calves.

We found that keeping animals continuously in their pens without grazing represents a risk factor for *Map*. Indeed, *Map* is able to survive up to 1 year in sun-protected areas such as animals' premises (Whittington, Marshall, Nicholls, Marsh, & Reddacliff, 2004). Moreover, the concentration of *Map* per gram of faeces in sheep clinically diseased, reaches up to 10^8 (Reddacliff, Eppleston, Windsor, Whittington, & Jones, 2006).

In conclusion, Ptb is a problem in several countries because it has huge health, economic and social impacts. Further investigations are needed to rank sheep Ptb in Tunisia among other diseases and to assess the main risk factors using a larger nation-wide sampling. If associated to pathological and bacteriological test, serology is the cheapest and easiest tool and would help characterising the epidemiological status of sheep flocks in an attempt to pave the way towards establishing specific control programmes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

MKK: wrote the paper, participated to the laboratory analyses. RR and LS: performed field sampling and participated to the laboratory analyses. AA: contacted farmers and participated in field visit. MR: funded part of the work and commented the manuscript. BM: monitored the study helped in study design and corrected the manuscript.

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