ELSEVIER

Small Ruminant Research 29 (1998) 13–19

Small Ruminant Research

Diagnosis and epidemiology of *Brucella ovis* infection in rams

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Accepted 12 September 1997

Abstract

The relationship between clinical, serological and bacteriological diagnostic criteria have been determined in *Brucella ovis* culture-positive and *Brucella* free control rams and also in 110 field rams belonging to 13 different flocks, in which an epidemiological survey was conducted. In control rams, a direct relationship was found among the existence of positive bacteriological results and the presence of a positive serological response in the gel diffusion (GD) and complement fixation (CF) tests for *B. ovis*. In field rams, the presence of macroscopic testicular alterations (MTA) caused by *B. ovis* was correlated with bacteriological and serological results. The percentage of reactors and the percentage of rams showing MTA was not related to the size of flocks nor to the ratios of ram/ewes. The prevalence of *B. ovis* infection was significantly higher in rams from imported breeds than in those from local ones. Moreover, the younger and the oldest rams showed the lowest prevalence of *B. ovis* infection, but the age of rams did not influence the prevalence of MTA not caused by *B. ovis*.

Keywords: Ram epididymitis; *Brucella ovis*; Diagnostic tests; Epidemiology

1. Introduction

Brucellosis is an important disease of animals produced by bacterial species included in the genus *Brucella*, some being zoonotic. *Brucella ovis* is a non-zoonotic, stable rough species that produces a clinical or subclinical chronic disease of sheep characterised by testicular alterations and subsequent low fertility in rams and occasional abortions in ewes. The existence of other bacteria causing clinical epididymitis makes the diagnosis of this disease difficult by testicular palpation alone (Blasco, 1990). The bacteriological diagnosis, if positive, is the only incontestable evidence of the disease. However, since infected rams may show intermittent or no excretion of *B. ovis* (Worthington et al., 1985), the bacteriological examination of semen samples is not a practical and reliable means for diagnosis in large-scale programs. Serological diagnosis is more appropriate for this purpose. A large variety of techniques have been applied to detect antibodies to *B. ovis*, but the most widely used are the gel diffusion (GD) and the complement fixation (CF) tests (Blasco, 1990).

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00921-4488/98/$19.00
PII S0921-4488(97)00108-9

Despite some contradictory evidence, a number of epidemiological factors are considered of importance in the transmission and prevalence of this disease (Murray, 1969; Blasco and Barberán, 1990). However, the effect of age and breed on susceptibility to infection remains a matter of controversy (Blasco, 1990). The aim of this work was to examine the relationship of the clinical, serological and bacteriological findings in *B. ovis* infected rams and to determine the relevance of several epidemiological factors on the prevalence of both *B. ovis* infection and macroscopic testicular alterations (MTA) in rams in Catalonia.

2. Material and methods

2.1. Animals

A total of 42 serum samples from *B. ovis* culture-positive rams and 53 from *Brucella* free rams, all with known clinical status and belonging to the collection of the Unidad de Sanidad Animal, SIA/DGA (Spain), were used as controls to evaluate the sensitivity and specificity of the serological tests used. Moreover, a total of 110 rams from 13 different sheep flocks from Catalonia (NE of Spain) were selected to conduct an epidemiological study. An inquiry was carried out to characterise each flock according to size, ratio of rams/100 ewes, and breed and age of rams. Each ram was examined by genital palpation to detect macroscopic testicular alterations (MTA), electroejaculated (Ruakura ram probe apparatus, Alfred Cox Surgical, UK) to obtain semen samples for bacteriological purposes, and blood samples obtained for serological testing.

2.2. Bacteriological tests

Swabs taken after electroejaculation from the preputial cavity of each ram were smeared on two plates of modified Thayer–Martin medium (Brown et al., 1971). Plates were incubated in 10% CO_2 for at least seven days at 37°C, and the suspected colonies identified by gram staining, urease, oxidase and acriflavine tests. Definitive identification was performed by phage R/C sensitivity, and serum and CO_2 requirements (Alton et al., 1976). A total of 26 out of 110 rams gave a positive culture for *B. ovis*.

No tests were conducted to determine the existence of pathogens other than *Brucellae*.

2.3. Antigen extraction and characterisation

The hot saline (HS) antigen extract was prepared from the CO_2-independent *B. ovis* REO 198 strain, grown in tryptic soy broth–yeast extract as described previously (Marín et al., 1989). Briefly, wet cells were suspended in saline (10% wt./vol.), and extracted at 100°C for 15 min. After removal of the cell debris by centrifugation ($15{,}000 \times g$; 15 min), the supernatant was dialysed against distilled water and ultracentrifuged ($100{,}000 \times g$; 6 h), and the sediment was suspended in distilled water and freeze-dried; it constituted the HS extract (Díaz and Bosseray, 1973). It has been shown previously that these extracts contain 45–65% protein, and 40–55% rough lipopolysaccharide (R-LPS) (Riezu-Boj et al., 1990).

2.4. Serological tests

The CF test was performed on microplates using the warm procedure with serum dilutions made in veronal buffer (Alton et al., 1976). Optimal antigen concentrations and test conditions were determined previously by titration with a panel of positive and negative control sera. Sera showing 50% or less hemolysis in the 1/2 or higher dilutions were considered as positive. In the final test, the HS antigen was used at 75 μg/ml in veronal buffer.

The GD test was carried out on petri plates covered with 3 mm of 1.1% noble agar (Difco Laboratories, Detroit, MI, USA), 10% NaCl in borate buffer (0.1 M, pH 8.3). The slides were incubated at room temperature and readings were taken after 24, 48 and 72 h. The HS antigen was used at 0.3 mg/ml in distilled water, and this optimal concentration was determined previously by titration against the panel of positive and negative control sera.

Before being applied to the field samples, the serological tests were standardised with the sera from the 42 positive and 53 negative control animals. The sensitivity and specificity of both serological tests were calculated as described by Worthington et

al. (1984). The GD test detected 91.8% of 61 culture-positive control rams showing 100% specificity with the 53 *Brucella* free controls. The sensitivity and specificity values for the CF test were 91.8% and 100%, respectively. Two sera were GD-negative but CF-positive, and one GD-positive but CF-negative. Accordingly, when considering the combined results in both tests, a total of 95.1% of 42 positive control sera were detected. Therefore, the serological results obtained with the field sera are always referring to the combined GD + CF results.

2.5. Statistical analysis

The MacNemar test was used to determine the differences between the clinical, bacteriological and serological results. The χ^2 contingency test was used to determine the significance of the different epidemiological factors on the prevalence of both *B. ovis* infection and TMA (Remington and Schork, 1979; Steel and Torrie, 1985).

3. Results

3.1. Serological, bacteriological and clinical results

Comparison of the clinical, serological and bacteriological results in control and field rams, and their statistical significance using the MacNemar test, are presented in Table 1. When considering the control rams, the results of serological tests were similar to bacteriological results. In these rams, the presence of MTA was not significantly related to positive serological or bacteriological results. However, the serological and bacteriological results were significantly different when testing the field rams, and, in these animals, the presence of MTA in serologically positive rams was directly related to a positive bacteriological result. Moreover, the positive results of semen culture in rams showing MTA ($n = 16$) were similar to that obtained in the GD test ($n = 14$), but were under-represented in culture-positive rams with a positive (CF-test) ($n = 26$).

Table 1
Comparison of the clinical, serological and bacteriological results in control and field rams

			Serological tests						MTA SER +	
			GD		CF		GD + CF			
			+	−	+	−	+	−	+	−
Control rams (95)	Culture	+	40 2.00[a]	2 (NS)	41 1.00	1 (NS)	42 0.00	0 (NS)	17 25.00[d]	25
		−	0	53	0	53	0	53	0	0
	MTA CULT +	+	15 19.5[d]	2	17 24[d]	0	17 25[d]	0		
		−	25	0	24	1	25	0		
Field rams (110)	Culture	+	24 10.89[d]	2	26 28.00[d]	0	26 30.00[d]	0	16 2.28 (NS)	10
		−	16	68	28	56	30	54	18	12
	MTA CULT +	+	14 2.00 (NS)	2	16 10.00[c]	0	16 10.00[c]	0		
		−	0	0	10	0	10	0		

GD: gel diffusion test; CF: complement fixation test; MTA: macroscopic testicular alterations; CULT +: positive culture for *B. ovis*; SER +: positive results in gel diffusion test and/or complement fixation test; MTA SER +: macroscopic testicular alterations caused by *B. ovis*; MTA CULT +: macroscopic testicular alterations caused by *B. ovis*.

[a] χ^2 values obtained by McNemar test and their statistical significance.

NS: not significant.

[b] $P < 0.05$; [c] $P < 0.01$; [d] $P < 0.001$.

Table 2
Relationship between flock size and bacteriological, serological and clinical results

Number of sheep in the flock	Size	*n*	CULT + (%)	SER + (%)	MTA + SER + (%)	MTA + SER − (%)
50–400	Small	20	$6^{a,b}$ (30.0)	7^{a} (35.0)	5^{a} (25.0)	4^{a} (20.0)
401–800	Medium	23	9^{a} (39.1)	15^{b} (65.2)	8^{a} (34.7)	7^{a} (30.4)
> 800	Large	67	11^{b} (16.4)	34^{ab} (50.7)	21^{a} (31.3)	12^{a} (17.9)
Total		110	26 (23.6)	56 (50.9)	34 (30.9)	23 (20.9)

CULT +: positive culture for *B. ovis*; SER +: positive results in gel diffusion test and/or complement fixation test; MTA + SER +: presence of macroscopic testicular alterations caused by *B. ovis*; MTA + SER −: presence of macroscopic testicular alterations not due to *B. ovis*.
Two identical letters in the same column correspond to non-significantly different results, and two different letters to significantly different results.

3.2. Epidemiological results

The bacteriological, serological and clinical results according to flock size are presented in Table 2. The percentages of bacteriologically positive rams were always lower than the percentages of clinically affected or serologically positive rams, independent of flock size. The percentage of reactors seemed to increase according to flock size, but significant differences ($P < 0.05$) were obtained only between small- and medium-sized flocks. The prevalence of MTA, caused by *B. ovis* or not, was not dependent on the flock size.

The prevalence of both MTA and *B. ovis* infection according to the breed of rams is presented in Table 3. The percentages of both serologic reactors and rams showing MTA caused by *B. ovis*, were significantly higher ($P < 0.01$ and $P < 0.001$, respectively) in imported breeds than in local rams. The percentage of seronegative but MTA affected rams was not significantly different between the imported or native breeds. The prevalence from both MTA and *B. ovis* infection according the age of rams is presented in Table 4. No clear significant differences were obtained among the different age classes. However, when comparing a group formed by rams from less than 2 years and over 5 years, and a second group including rams from 2 to 5 years, a significant difference was obtained with respect to the bacteriological ($P < 0.05$) and serological ($P < 0.05$) results. Moreover, significant differences among both ram classes were obtained on the preva-

Table 3
Prevalence of macroscopic testicular alterations and *B. ovis* infection according the breed of rams

Origin	Breed	*n*	CULT + (%)	SER + (%)	MTA + SER + (%)	MTA + SER − (%)
Local	Berberina	21	16^{a}	32^{a}	16^{a}	18^{a}
	Cartera	39				
	Ripollesa	16	(21.0%)	(42.1%)	(21.0%)	(23.6%)
		(Total = 76)				
Imported	Berrichon	8				
	Charolaise	2	10^{a}	24^{b}	18^{b}	5^{a}
	Ile de France	6				
	Lacaune	10	(29.4%)	(70.5%)	(52.9%)	(14.7%)
	Rouge de l'ouest	8				
		(Total = 34)				

CULT +, positive culture for *B. ovis*; SER +: positive results in gel diffusion test and/or complement fixation test; MTA + SER +: presence of macroscopic testicular alterations caused by *B. ovis*; MTA + SER −: presence of macroscopic testicular alterations not due to *B. ovis*.
Two identical letters in the same column correspond to non significantly different results, and two different letters to significantly different results.

Table 4
Prevalence of macroscopic testicular alterations and *B. ovis* infection according the age of rams

Age (yr)	*n*	CULT + (%)	SER + (%)	MTA + SER + (%)	MTA + SER − (%)
1	25	4 (16.0%)	10 (40.0%)	5 (20.0%)	4 (16.0%)
2	26	8 (30.7%)	18 (69.2%)	16 (61.5%)	4 (15.3%)
3	12	4 (33.3%)	6 (50.0%)	2 (16.6%)	3 (25.0%)
4	16	4 (25.0%)	9 (56.2%)	4 (25.0%)	6 (37.5%)
5	16	6 (37.5%)	9 (56.2%)	6 (37.5%)	4 (25.0%)
>5	15	0 (0.0%)	4 (26.6%)	1 (6.6%)	5 (33.3%)
<2 and >5	40	4[a] (10.0%)	14[a] (35.0%)	6[a] (15.0%)	9[a] (22.5%)
2–5	70	22[b] (31.4%)	42[b] (60.0%)	28[b] (40.0%)	17[a] (24.2%)

CULT +: positive culture for *B. ovis*; SER +: positive results in gel diffusion test and/or complement fixation test; MTA + SER +: presence of macroscopic testicular alterations caused by *B. ovis*; MTA + SER −: presence of macroscopic testicular alterations not due to *B. ovis*.
Two identical letters in the same column correspond to non-significantly different results, and two different letters to significantly different results.

lence of MTA due to *B. ovis* ($P < 0.01$), but not on the prevalence of MTA due to causes other than *B. ovis*.

4. Discussion

It has been generally accepted that the diagnosis of *B. ovis* infection can be made using clinical, bacteriological and serological criteria. However, the clinical examination of rams to determine the presence of MTA is of limited value for the diagnosis of *B. ovis* infection because of the existence of infected rams without clinical signs (Blasco et al., 1987; Blasco and Barberán, 1990). Moreover, pathogens other than *B. ovis* are frequently responsible for MTA in rams (Rodolakis and Bernard, 1977; Cox et al., 1977; Delong and Waldhalm, 1979; Webb et al., 1980), limiting the usefulness of the clinical examination of ram genitalia for the diagnosis of brucellosis. In our work, the existence of MTA caused by *B. ovis* was closely related to bacteriological and serological results in field rams, but not in control ones (Table 1). By contrast, serological and bacteriological results were more closely related in control rams than in field ones (Table 1). The number of field rams seropositive was significantly higher than that of rams bacteriologically positive. This is consistent with previously published data (Blasco, 1990) reporting that a large proportion of seropositive rams do not show seminal excretion. In addition, our field rams were sampled once for semen culture and intermittent *B. ovis* seminal excretion has been reported in infected rams (Worthington et al., 1985). Finally, *B. ovis* may be isolated only from sites other than the genitalia or semen in some infected but seropositive rams (Blasco, 1990). The two serological tests used have been reported to be suitable for the diagnosis of *B. ovis* infection (Marín et al., 1989; Ficapal et al., 1995). Being simpler than the CF test, the GD has been recommended as the choice for the serological diagnosis (Marín et al., 1989). However, this serological test is not exempt from problems. First, once solubilised for use in this test, the HS antigen diminishes its efficacy over time, and it is difficult to maintain in this solubilised form (Ficapal, Moriyon and Blasco, unpublished results). Second, some infected rams are GD-negative but CF-positive and vice versa, and accordingly, the highest percentage of infected rams is usually detected by the combination of both tests (Marín et al., 1989). In our study, the individual sensitivity of both GD and CF tests in control rams was 91.8%, while the combination of results in both tests increased sensitivity to 95.1%.

It is accepted that the prevalence of bovine brucellosis due to *B. abortus* increases according to the herd size (Crawford et al., 1990). However, there is no available information concerning the relationship between the flock size and the prevalence of both *B. ovis* and MTA in rams. As in bovine brucellosis, the

serological prevalence seemed to increase in accordance with the size of flocks (Table 2). In fact, the percentage of reactors was significantly lower in small flocks than in medium-sized ones ($P < 0.05$). Surprisingly, no significant differences were obtained when comparing small or medium flocks with large-sized ones. This could be explained, at least in part, by the fact that owners of large flocks could have a higher level of technical expertise and better management practices than those from small- or medium-sized flocks. The prevalence of MTA due either to brucellosis (that is, occurring in seropositive animals) or not, did not increase according to the flock size (Table 2).

To the best of our knowledge, there is no information available concerning the relationship between the ratio of ram/ewe on the prevalence of MTA and *B. ovis* infection. In our epidemiological survey, this ratio was not correlated with the bacteriological, serological or clinical results (data not shown).

There are many references showing that the susceptibility to both MTA and brucellosis may vary among breeds of rams. The Merino breed appears to be less frequently infected than British breeds in the same environment (Watt, 1970; Blasco, 1990). Some Spanish native and Merino-derived breeds are less sensitive to MTA and ovine brucellosis than other imported European breeds (Marín et al., 1986). In our study, the prevalence of MTA due to *B. ovis* was significantly lower in native breeds than in imported ones. By contrast, the prevalence of MTA not due to brucellosis was not significantly different among imported or native breeds. Moreover, the rams from native Berberina, Cartera and Ripollesa breeds showed a significantly lower prevalence of brucellosis than those from imported breeds (Table 3). Although genetic resistance to the disease could be important in explaining these results, it has been suggested that this different susceptibility could be related to differences in growth rates and sexual precocity and activity (Marín et al., 1986), that are usually higher in imported breeds. Finally, another explanation of these differences could be found in the sanitary origin of animals. Those rams from *Brucella*-free countries that are imported into affected countries without applying any prophylactic measures, could be highly susceptible. This would be true for other infectious causes of MTA as well.

Whether age affects the susceptibility to *B. ovis* infection and MTA is controversial. The *B. ovis* infection has been demonstrated in very young rams, suggesting that animals at, or soon after puberty, could be highly susceptible to *B. ovis* (Giaufret and Sanchis, 1974). However, since venereal transmission seems to be the main way of spreading brucellosis (Lawrence, 1961; Blasco, 1990), adult animals are more likely to be naturally infected. In fact, it has been reported that the incidence of both testicular alterations and brucellosis increases with age, and relates to the sexual experience of the animals (Marín et al., 1986; Blasco, 1990). In our management conditions in Catalonia, the youngest and the oldest rams were significantly less affected by *B. ovis* infection than the intermediate age ram classes (Table 4). The youngest animals, with little sexual experience, and the oldest, with diminished sexual activity, may therefore be less exposed to infection.

5. Conclusions

The prevalence of *B. ovis* infection in Catalonia is mostly dependent on the breed and age of rams. The number of rams affected by brucellosis was significantly higher in imported rams than in local ones. Moreover, the youngest (< 2 years) and oldest (> 5 years) animals were found to be significantly less affected by brucellosis than those from intermediate age classes. Finally, none of the management factors studied (flock size, breed and age) had a clear influence on the prevalence of MTA due to causes other than brucellosis.

Acknowledgements

We thank the DARP from the Generalitat de Catalunya for its contribution to this work. Heads and staff members from the Departamento de Microbiología from the Universidad de Navarra and the Unidad de Sanidad Animal from the SIA/DGA are gratefully acknowledged. Particular thanks to A. Gil, M.A. Pallaruelo and all the owners of surveyed flocks, for their assistance with the collection of the blood and semen samples.

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