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EVALUATION OF ROSE BENGAL TEST, GEL IMMUNODIFFUSION WITH NATIVE HAPTEN, AND INDIRECT ELISA IN DIAGNOSIS OF BRUCELLOSIS IN INFECTED AND VACCINATED SHEEP

(With 2 Tables)

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تقييم اختبار الروزبنجال والأنتشار المناعى فى الجل باستخدام الهابتين المحلى وأختبار الاليزا الغير مباشر فى تشخيص البروسيلا فى الاغنام المصابة والمحصنة بالبروسيلا

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اجريت دراسة لتقييم اختبار الروزبنجال والأنتشار المناعى فى الجل باستخدام الهابتين المحلى وأختبار الاليزا الغير مباشر فى تشخيص البروسيلا فى الاغنام المصابة والمحصنة بالبروسيلا وقد وجد ان تخفيف السيرم: انتيجن الروزبنجال بنسبة 1:3 بعطى نتيجة ايجابية بنسبة حساسية 100%. وبفحص 49 حيوان مصابة بالبروسيلا بهذة الاختبارات فى الاغنام وجد ان الروزبنجال والاليزا الغير مباشر تعطى نتيجة 100% ايجابية بينما اختبار الأنتشار المناعى فى الجل باستخدام الهابتين المحلى يعطى 93.8% ايجابية. اختلافات كثيرة ظهرت عند استخدام هذه الاختبارات فى اختبار السيرم لهذة الاغنام فى المناطق المتوطن بها مرض البروسيلا وعند استخدام الاختبارات فى الاغنام المحصنة تحت الجلد وفى العين وجد ان هذه الاختبارات تكون سلبية عند 12 شهر و6 شهور من تاريخ التحصين بالتتابع. وقد استفدنا من هذه الدراسة ان استخدام الروزبنجال أو الاليزا مع اختبار الأنتشار المناعى فى الجل باستخدام الهابتين المحلى يعطى فرصة أحسن فى تشخيص البروسيلا فى الاغنام المحصنة والمصابة.

SUMMARY

Three serological assays were evaluated for the diagnosis of brucellosis in sheep: the Rose Bengal plate test as a screening test (RBPT), agar gel immunodiffusion with Native Hapten test (AGID-NH) and an indirect enzyme-linked immunosorbent assay (iELISA). For optimal sensitivity,

RBPT had to be used with sera: antigen at a 3:1 dilution. With the sera from *B. melitensis* culture-positive sheep, the sensitivity was 100% for RBPT, and iELISA; while, it was 93.8% for AGID-NH test. On the other hand specificity was 100% when testing the sera from 145 brucella-free sheep. Larger discrepancies among the results of the serological tests were obtained with sera from sheep of areas where brucellosis is endemic. Sheep vaccinated with *B. melitensis* Rev.1 subcutaneously and conjunctively showed negative result in all tests after twelve and six months respectively. The proportion of sheep giving a positive reaction after vaccination decreased faster in AGID-NH test than in other tests. The using of RBPT or ELISA with combination with AGID-NH is the best choice for diagnosis of infected and vaccinated sheep.

Key words: *Brucella, iELISA, AGID-NH*

INTRODUCTION

Brucellosis is an infectious disease of worldwide importance in domestic ruminants, and the causative bacteria are transmitted to humans through contact with infected livestock or by consumption of contaminated dairy products. Because of the economic importance of cattle in developed countries, means for *B. abortus* diagnosis and prophylaxis have been widely investigated (Alton *et al.,* 1988; Nicoletti, 1990; Plommet and Fensterbank, 1984; Zundel *et al.,* 1992) and several serological tests developed for cattle brucellosis have been found useful for the diagnosis *of B. melitensis* infection in sheep (Alton, 1990; Diaz-Aparicio *et al.,* 1993; Jimenez de Bagues *et al.,* 1992).

The control of sheep brucellosis is usually based on vaccination, serological testing, and culling the positive. Until now, the best vaccine available has been the smooth *B. melitensis* Rev.1 strain. Although this strain is useful, it does not give 100% protection, and it induces a strong antibody response to the S lipopolysaccharide (S-LPS).

Since S-LPS is the most relevant antigen in conventional serological tests such as the Rose Bengal plate test (RBPT) and the complement fixation test (CFT) (Alton *et al.,* 1988), it is not surprising that Rev.1 vaccination interferes with serological diagnosis. Even so, conventional serological diagnosis requires the use of screening and confirmatory tests such as RBPT and CFT, respectively. However, the use of these two tests does not result in 100% sensitivity and specificity (Blasco, 1994 b). To reduce these restrictions in the use of the vaccine

and to facilitate serological diagnosis, alternative assays have been investigated, including tests that detect antibodies to proteins (Dı´az *et al.,* 1984; Zygmunt, *et al.,* 1994; Debbar *et al.,* 1995; Letesson, *et al.,* 1997) and to the S-LPS-related native hapten (NH) polysaccharide (Blasco *et al.,* 1984 a; Jime´nez de Bagu¨e´s *et al.,* 1992; Dı´az-Aparicio *et al.,* 1993; Dı´az-Aparicio *et al.,* 1994).

The aim of the present work was to standardize the most used conventional test (i.e., RBPT) for sheep brucellosis, to compare the AGID-NH test with the iELISA using control sera from infected and brucella-free sheep and sera from sheep from areas where brucellosis is endemic; and to evaluate the interference of the postvaccinal serological response in the previously mentioned tests.

MATERIALS and METHODES

A- Sera separated from the collected blood samples of the following animals were used:-

- **Group I:** *B. melitensis*-infected sheep. Forty nine sheep with field vaccination had not been used. No previous selection of the animals was made on the basis of any serological test.
- **Group II:** Vaccinated sheep. A young sheep (3 months old) were vaccinated subcutaneously (59 animals) and conjunctively (34 animals) with 10^9 CFU of *B. melitensis* Rev.1 and blood samples were collected every 2 months after subcutaneous and conjunctival vaccination till be negative serologically.
- **Group III:** Brucella-free sheep. One hundred forty five sheep were from two flocks free of brucellosis in which vaccination had not been performed.
- **Group IV:** non-vaccinated sheep from areas in which brucellosis is endemic. Two hundred thirty sheep were from an area where brucellosis is endemic and no vaccine used.

B- Serological tests: (1) RBPT was performed with a 3:1 of proportion serum to antigen for optimal sensitivity (Blasco *et al.,* 1994).

(2) Agar gel immunodiffusion test for detecting NH-precipitating antibodies (AGID-NH) was performed with 1% Noble agar (Difco Laboratories, Detroit, Mich.) gels in 10% NaCl–0.1 M NaOH-H3BO4 (pH 8.3) with 20 ul of serum and the antigen wells set 3 mm apart. The antigen was an NH (Brucella Lab. CITA, Zaragoza, Spain) rich *B.melitensis* 16 M hot-water extract in which the NH precipitation ring

is characteristic (Dı´az *et al.,* 1981; Moreno *et al.,* 1981; Dı´az *et al.,* 1984; Arago´n *et al.,* 1996).

(3) iELISA was performed with a crude *B.melitensis* S-LPS preparation (Dı´az *et al.,* 1981; Jime´nez de Bagu¨e´s *et al.,* 1992; Arago´n *et al.,* 1996; Alonso-Urmeneta *et al.,* 1998) and peroxidase-conjugated protein G, and the results were expressed as the percentage of the optical density with respect to a strongly positive control serum (Jime´nez de Bagu¨e´s *et al.,* 1992; Dı´az-Aparicio *et al.,* 1994; Alonso-Urmeneta *et al.,* 1998).

The sensitivity and specificity of the tests were calculated with respect to the infected and brucella-free groups as described by Jones *et al.* (1973).

RESULTS

The sera from the *B.melitensis* infected and *brucella*-free animals showed optimal sensitivity with 1:50 of the serum dilution, with these sera, the iELISA completely discriminated the sera from the *brucella*-free and *B.melitensis*-infected populations (100% sensitivity and specificity). The RBPT had performed as currently recommended failed to detect 4 of the 49 infected sheep (91.8% sensitivity). In contrast, when the proportion of serum was increased (25 µl of antigenic suspension and 75 ul of serum), RBPT gave no false-negative results (Table 1) and showed 100% sensitivity. The two tests were more sensitive than the AGID-NH test (93.8%). The AGID-NH test and RBPT showed 100% specificities for sera from *brucella*-free animals.

On the other hand, the results obtained with sera from Sheep from an area in which brucellosis is endemic (Table 1) showed larger discrepancy than the results obtained with sera from infected and free sheep. The RBPT and iELISA showed sensitivity of 35.6% and 33.0% respectively, while the AGID-NH test showed a sensitivity of only 18.2%.

Table 2 shows the interference of the post-vaccinal serological response in the above serological tests. The specificities of the tests (i.e., the percentage of vaccinated animals that tested negative and, therefore, would not be misdiagnosed as infected) varied depending upon the route of vaccination. In subcutaneous route, the AGID-NH test was 100% specific at six months following vaccination, while the RBPT and iELISA showed specificity of 32.8% and 24.1% respectively. On the

other hand, the AGID-NH test had a faster specificity of 100% at four months post-vaccination using the conjunctival rout. The RBPT and iELISA had specificity of 85.3% and 76.5% respectively at the same period.

Table 1: Results of serological tests of sera from infected sheep and sheep of endemic areas.

Tested Group		Status	TEST No. of serum samples positive in		
Group No.	Animal No.		RBPT	ELISA	AGID-NH
I	49	Infected	49	49	44
Sensitivity			100%	100%	93.8%
II	230	Endemic	82	76	42
Sensitivity			35.6%	33.0%	18.2%

Table 2: Specificities of serological tests for brucellosis for sera from *B.melitensis* Rev1-vaccinated sheep

Age of vaccination/m	Rout	Sampling /month(b)	No. of Sheep	% Specificity of (a)		
				RBPT	AGID-NH	iELISA
3	Subcutaneous	2	59	8.5	30.5	3.4
		4	58	15.5	82.8	10.3
		6	58	32.8	100	24.1
		8	55	63.6	100	58.1
		10	48	89.6	100	87.5
		12	47	100	100	100
3	Conjunctival	2	34	32.4	88.2	26.5
		4	34	85.3	100	76.5
		6	34	100	100	100

a= Specificity is defined as the percentage of vaccinated animals that are negative in a given test.
b= Number of months after vaccination

DISCUSSION

In the present work, the above tests have been evaluated using sera from sheep with brucellosis proved by bacteriological isolation of *B.melitensis* biotype 3 from their tissues and milk samples. The results show that they are all potentially useful for the diagnosis of *B. melitensis* infection in sheep. However, the RBPT had to be modified by increasing the serum/antigen ratio to reach 100% sensitivity. This confirms (Blasco *et al.,* 1994 a) that present guidelines for the standardization of the RBPT are not adequate for sheep and goat brucellosis. Therefore, the lower sensitivity reported before for RBPT (Falade, 1978; Waghela *et al.,* 1980) is possibly due to the use of sera from animals of unknown infectious status and / or, the use of the standard method.

The sera from the *B. melitensis* infected and *brucella*-free sheep showed that iELISA yielded optimal sensitivity and specificity with the 1:50. Serum dilution. With these sera, the iELISA completely discriminated the sera from the *Brucella*-free and *B. melitensis*-infected populations (100% sensitivity and specificity). The assay described here with S-LPS is not essentially different from some indirect ELISAs used for animal brucellosis (Alonso-Urmeneta *et al.,* 1988; Jime´nez de Bagu¨e´s *et al.,* 1992; Nielsen *et al.,* 1988; Wright and Nielsen, 1990). But several methodological aspects deserve further comments. It was found that the protein G conjugate reduced the reactivity of the negative sera (i.e., it increased the ELISA specificity). In cattle brucellosis, Wright and Nielsen (1990) have found that an anti-IgGl monoclonal conjugate increases the specificity of the indirect ELISA (with S-LPS) with respect to conjugates prepared with polyclonal antibodies to IgG heavy and light chains or with a monoclonal antibody to the light chain.

When sera from unvaccinated sheep in areas where brucellosis is endemic were tested, a large disagreement was found between the AGID-NH test and RBPT. There is evidence that sera with specific Ig M but without significant levels of Ig G are positive in RBPT and negative in AGID-NH test (Alonso-Urmeneta *et al.,* 1988). Thus, those discrepant results could correspond to animals that were in the early stages of the host-parasite interaction before the infection was (or was not) established.

Since live attenuated vaccines are powerful tools in the eradication of brucellosis, the interference of the postvaccinal response in the serological diagnosis is a major problem and should be considered whenever serological tests are evaluated. The specificities of all tests were generally higher when the sera of conjunctively vaccinated sheep

were tested (Jime´nez de Bagu¨e´s *et al.,* 1992). Jones *et al.* (1973) reported that the RBPT test and CFT become negative by the 4th month after subcutaneous vaccination of goats with 10^9 CFU of Rev.1, and the more protracted positive serological response found in our work is likely to be due to the use of a modified RBPT. Also, our results suggest that ELISA with S-LPS would not outperform RBPT after subcutaneous vaccination. Jones *et al.* (1973) also observed that vaccination with a much reduced dose (5×10^4 CFU) of Rev.1 shortened the postvaccinal response to less than 2 months. However, there is evidence that vaccination with reduced doses confers a less solid immunity (Elberg, 1981), and therefore, the conjunctival route (Fensterbank *et al.,* 1987; Zundel *et al.,* 1992) seems a better alternative. The results presented here confirm for RBPT (Fensterbank *et al.,* 1987), and illustrate for iELISA, and the AGID-NH tests, that the serological response after vaccination of sheep is considerably reduced by this route, with the AGID-NH test as the test that become negative faster. This reduction of the postvaccinal serological response by the use of the conjunctival route has also been shown in cattle (*B. abortus* 19 vaccine) and sheep (*B. melitensis* Rev.1 vaccine) ((Jime´nez de Bagu¨e´s *et al.,* 1992).

It is clear that a relatively simple test such as the AGID-NH test was specific in diagnosis of brucellosis in sheep. Immunochemical studies have shown that the NH and the O- polysaccharide of the S-LPS (which is the serologically relevant section of S-LPS) have similar structures and epitopic densities (Arago´ *et al.,* 1996; Dı´az-Aparicio *et al.,* 1993). In fact, antibodies to the NH can be absorbed with S-LPS (Alonso-Urmeneta *et al.,* 1988). However, precipitation tests with S-LPS do not show the sensitivity and specificity of similar tests performed with NH (Dı´az *et al.,* 1984; Dı´az *et al.,* 1981; Moreno *et al.,* 1981), although NH and S-LPS yield similar results in both iELISA (Alonso-Urmeneta *et al.,* 1998; Dı´az-Aparicio *et al.,* 1994) and passive hemagglutination (Alonso-Urmeneta *et al.,* 1988).

To explain these apparently contradictory observations, we have proposed (Alonso-Urmeneta *et al.,* 1988) that the higher specificities of the precipitation tests with NH result from two sets of factors. First, the dispersed state of the low-molecular-weight NH (Arago´n *et al.,* 1996) in solution, as opposed to the highly aggregated S-LPSs, may be relevant in explaining their different behavior in precipitation tests. Second, if low-affinity antibodies are predominant after vaccination, the higher threshold affinity of precipitation tests compared to that of iELISAs (Peterfy *et al.,* 1998) may explain why NH fails to react with sera from

vaccinated animals in the former but not in the latter assay (Gabriela *et al.*, 2009).

The results of this work have practical implications concerning the use of the tests evaluated. In the absence of vaccination, the iELISA and the much less sophisticated RBPT (standardized and performed as described (Blasco *et al.*, 1994 a) should be the tests of choice because of their very high sensitivities. When vaccination was implemented, no single test simultaneously afforded 100% sensitivity and specificity **(**Dabdoob *et al.*, 2000).

However, screening with either the iELISA or the RBPT followed by confirmation by means of the AGID-NH test would afford the best combination of sensitivity and specificity, the latter seems to be the simplest choice for the diagnosis of sheep brucellosis when Rev.1 vaccination is implemented.

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