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Review article

Brucella melitensis infection in sheep: present and future

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Abstract – Sheep brucellosis, a zoonosis mainly due to *B. melitensis* (biovar 1, 2 or 3), remains widespread world-wide. Pathologically and epidemiologically, the disease is very similar to *B. abortus* infection in cattle. The live *B. melitensis* Rev 1 strain is currently considered as the best vaccine available for the control of sheep brucellosis, especially when used at the standard dose by the conjunctival route. Used exhaustively in whole-flock vaccination programmes, it induces a great decrease in the prevalence in both sheep and human populations. The expensive test-and-slaughter strategy should be restricted to the lowest infected areas. Whenever possible, *Brucella* spp. should be isolated by culture using adequate selective media from uterine discharges, aborted fetuses, udder secretions or selected tissues, such as lymph nodes, testes or epididymides. Species and biovar identification is routinely based on cultural criteria, on lysis by phages and on simple biochemical and serological tests. The recently developed polymerase chain reaction methods provide additional means of detection and identification. Despite the high degree of DNA homology within the genus *Brucella*, several methods, including PCR-RFLP and Southern blot, have been developed which allow, to a certain extent, the differentiation between *Brucella* species and some of their biovars. While several ELISA tests have been developed recently, the rose bengal plate agglutination and complement fixation tests, based on the detection of anti-S-LPS antibody, are still recommended for screening flocks and individuals. However, these tests sometimes lack specificity or sensitivity. For pooled samples, there are no useful tests such as the milk ring test in cattle. The brucellin allergic skin test can be used as a screening or complementary test in unvaccinated flocks, provided that a purified, lipopolysaccharide (LPS)-free and standardized antigen preparation is used. © Inra/Elsevier, Paris

sheep brucellosis / *Brucella melitensis* / update / perspectives

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Résumé – Brucellose ovine à *Brucella melitensis* : présent et avenir. La brucellose ovine, zoonose principalement due à *Brucella melitensis* (biovar 1, 2 ou 3), demeure d'importance mondiale. Aux plans pathologique et épidémiologique, la maladie est similaire à la brucellose bovine. La vaccin vivant *B. melitensis* Rev 1 est actuellement le plus efficace pour le contrôle de l'infection, particulièrement s'il est utilisé à dose standard par voie conjonctivale. La vaccination généralisée des troupeaux, permet, lorsqu'elle est exhaustive, une réduction importante de la prévalence humaine et animale. La prophylaxie sanitaire stricte, qui est très coûteuse, doit quant à elle être réservée aux zones très peu infectées. Les *Brucella* doivent être recherchées, si possible, par culture sur milieu sélectif adapté, à partir des sécrétions utérines, de l'avorton, des sécrétions mammaires ou de certains tissus, nœuds lymphatiques, testicule ou épididyme. L'identification de l'espèce et du biovar est réalisée en pratique à partir de critères culturaux, de la lyse par les phages et au vu des résultats de tests biochimiques et sérologiques simples. Les méthodes PCR récemment mises au point sont un outil complémentaire pour la détection et l'identification de la bactérie. Malgré la forte homogénéité génomique au sein du genre, différentes méthodes, essentiellement PCR-RFLP et Southern blot, ont pu être développées, qui permettent, dans une certaine mesure, de différencier les espèces de *Brucella* et leurs biovars. Malgré le développement récent de diverses méthodes Elisa, l'épreuve à l'antigène tamponné (Rose-Bengale) et la fixation du complément, qui permettent la mise en évidence d'anticorps anti-LPS-S, demeurent les tests de référence pour le dépistage individuel ou de troupeau. Néanmoins, ces tests manquent parfois de spécificité ou de sensibilité. Il n'existe enfin aucun test de mélange, analogue au ring-test pour les bovins, qui soit efficace. L'épreuve cutanée allergique est un test utile tant pour le dépistage que pour la confirmation des tests sérologiques dans les troupeaux non vaccinés, pourvu que l'allergène utilisé soit standardisé et dépourvu de LPS-S.

brucellose ovine / *Brucella melitensis* / mise au point / perspectives

Plan

1. INTRODUCTION

Brucella are Gram-negative, facultative, intracellular organisms which cause serious diseases in both humans and animals. Sheep brucellosis (excluding *Brucella ovis* infection) is a zoonotic infection with important effects on both public health and animal production and is widespread in many areas of the world, particularly in Mediterranean countries. Sheep brucellosis is primarily caused by *B. melitensis*, and rarely by *B. abortus* (Luchsinger and Anderson, 1979; Garin-Bastuji et al., 1994) or *B. suis* (Paolicchi et al., 1993). In this review, we have attempted to include up-to-date knowledge on *B. melitensis* infection in sheep giving particular attention to the taxonomy of the organism, the epidemiology, the control and the diagnosis of the disease. Despite a considerable increase in knowledge in recent years, many aspects of brucellosis in sheep remain unknown, unclear or controversial.

2. TAXONOMY OF *BRUCELLA* SPECIES INVOLVED IN SHEEP BRUCELLOSIS

Considering their high degree of DNA homology (> 90 % for all species), *Brucella* have been proposed as a monospecific genus in which all types should be regarded as biovars of *B. melitensis* (Verger et al., 1985). Since this proposal has not yet met with complete agreement, the old classification of the genus (and relevant nomenclature) into six species, i.e. *B. melitensis*, *B. abortus*, *B. suis*, *B. neotomae*, *B. ovis* and *B. canis* (Corbel and Brinley Morgan, 1984), is the classification used world-wide. The first four species are normally observed in the smooth form, whereas *B. ovis* and *B. canis* have only been encountered in the rough form. Three biovars are recognised for *B. melitensis* (1–3), seven for *B. abortus* (1–6 and 9) and five for *B. suis* (1–5).

Species identification is routinely based on lysis by phages and on some simple biochemical tests (oxidase, urease, etc.). For *B. melitensis*, *B. abortus* and *B. suis*, the identification at the biovar level is currently performed by four main tests, i.e. carbon dioxide (CO_2) requirement, production of hydrogene sulphide (H_2S), dye (thionin and basic fuchsin) sensitivity, and agglutination with monospecific A and M anti-sera. Moreover, the recent development of a coagglutination test, using a pair of mAb-coated latex beads, directed against the rough lipopolysaccharide (R-LPS) and the 25 kDa outer membrane protein (Omp 25), respectively (Bowden et al., 1997), makes it possible to differentiate *B. ovis* from *B. canis* and the occasional rough isolates of the smooth *Brucella* species accurately. The phenotypic characteristics of the three species involved in sheep brucellosis, i.e. *B. melitensis*, *B. ovis* and occasionally *B. abortus*, are presented in *table I* (species) and *table II* (biovars).

Intermediate strains are occasionally found due to the instability reported for some of the phenotypic characteristics used for the current classification of *Brucella*. This situation sometimes impedes the identification of the species and their biovars. Therefore, the identification of stable DNA-specific markers is considered a high priority for taxonomic, diagnostic and epidemiological purposes.

Several methods, mainly PCR-RFLP and Southern blot analysis of various genes or loci, have been employed to find DNA polymorphism which would enable the molecular identification and typing of the *Brucella* species and their biovars (Allardet-Servent et al., 1988; Ficht et al., 1990, 1996; Halling and Zehr, 1990; Halling et al., 1993; Fekete et al., 1992b; Grimont et al., 1992, Herman and De Ridder, 1992; Bricker and Halling, 1994,

Table I. Differential phenotypic and molecular characteristics of *Brucella* species involved in sheep brucellosis.

Species	Oxidase	Urease	Lysis by phages[a]			PCR-RFLP patterns[b] of genes (restriction endonuclease)				
						Omp25	Omp2b		Omp31	dnaK
			Tb	Iz	R/C	(EcoRV)	(AluI)	(PvuII)	(Sau3a)	(EcoRV)
B. melitensis	+	+	–	+	–	NC[d]	P1	NC	P1	P1
B. ovis	–	–	–	–	+	P1[e]	P2	P1	NC	P2
B. abortus	+[c]	+[c]	+	+	–	P2	P1	NC	NA[f]	P2

[a] At the routine test dilution; [b] for each gene, the different patterns (P1 or P2) are defined by the number and/or size of restriction fragments; [c] usually positive; [d] NC: not cleaved; [e] *B. ovis* omp25 pattern is related to a short deletion of 36 bp in the gene; [f] NA: no amplification. *B. abortus* lacks the omp31 gene.

Table II. Biovar differentiation of *Brucella* species involved in sheep brucellosis.

Species	Biovar or colonial morphology	CO_2 requirement	H_2S production	Growth on dyes[a]		Agglutination in			
				Thionin	Basic Fuchsin	polyclonal sera anti-		latex-mAb anti-	
						A	M	R-LPS	omp25
B. melitensis	1	–	–	+	+	–	+	–	–
	2	–	–	+	+	+	–	–	–
	3	–	–	+	+	+	+	–	–
	Rough	–	–	+	+	–	–	+	+
B. ovis	Rough	+	–	+	–	–	–	+	–
B. abortus	1	+[b]	+	–	+	+	–	–	–
	2	+[b]	+	–	–	+	–	–	–
	3	+[b]	+	+	+	+	–	–	–
	4	+[b]	+	–	+	–	+	–	–
	5	–	–	+	+	–	+	–	–
	6	–	–	+	+	+	–	–	–
	9	+ or –	+	+	+	–	+	–	–
	Rough	+ or –[c]	+ or –[c]	+ or –[c]	+ or –[c]	–	–	+	+

[a] Dye concentration, 20 µg/mL in Blood Agar Base medium with 5 % of serum (1:50 000); [b] usually positive on primary isolation; [c] + or –, according to the original smooth type.

1995; Cloeckaert et al., 1995, 1996c; Mercier et al., 1996; Ouahrani et al., 1993; Ouahrani-Bettache et al., 1996; Vizcaino et al., 1997). Among these methods, detection of polymorphism by PCR-RFLP, since it is easier to perform and is less time-consuming when applied to large numbers of samples, is considered to have an advantage over Southern blotting.

Of all the DNA sequences investigated by PCR-restriction, the major outer-membrane protein (OMP) genes of *Brucella* are the most interesting as they exhibit sufficient polymorphism to allow differentiation between *Brucella* species and some of their biovars (Cloeckaert et al., 1996d). Studies of the RFLP patterns of two closely related genes, *omp2a* and *omp2b*, encoding and potentially expressing the *Brucella* spp. major OMP of 36 kDa (Ficht et al., 1988, 1989), showed that the type strains of the six *Brucella* species could be differentiated on this basis (Ficht et al., 1990). More recently using PCR-RFLP and a greater number of restriction enzymes, Cloeckaert et al. (1995) detected *Brucella* species-, biovar- or strain-specific markers for the *omp25* gene, encoding the *Brucella* 25 kDa major OMP (de Wergifosse et al., 1995), and for the *omp2a* and *omp2b* genes. The *omp31* gene (Vizcaino et al., 1996), encoding a major outer-membrane protein in *B. melitensis*, is also an interesting gene for the differentiation of *Brucella* members. Using a combination of *omp31* PCR-RFLP patterns and Southern blot hybridization, profiles of *Brucella* species were differentiated with the exception of *B. neotomae* which was indistinguishable from *B. suis* biovars 1, 3, 4 and 5. It was also shown that *B. abortus* lacks a large DNA fragment of about 10 kb containing *omp31* and its flanking DNA (Vizcaino et al., 1997).

More highly conserved *Brucella* genes may also be useful for taxonomic and epidemiological purposes, even if they detect less polymorphism than the OMP genes. In this respect, the *dna*K locus which allows the identification of *B. melitensis*, the main *Brucella* pathogen for sheep, is of particular interest. All *B. melitensis* biovars showed a specific PCR-RFLP pattern with *Eco*RV, consistent with the presence of a single site instead of two for the other *Brucella* species (Cloeckaert et al., 1996c).

A selection of PCR-RFLP patterns allowing the clear differentiation of *Brucella* species involved in sheep brucellosis is presented in *table I*. The electrophoretic analysis of PCR-amplified *omp25* and relevant *Eco*RV-restriction products for *B. abortus*, *B. melitensis* and *B. ovis* is illustrated in *figure 1*. The PCR patterns allow the differentiation of *B. ovis*, the *omp25* amplicon of which is shorter due to a deletion of 36 bp *(figure 1a)*, and the *Eco*RV-RFLP patterns the differentiation of *B. melitensis* that lacks the *Eco*RV site *(figure 1b)*.

Clearly, taxonomic knowledge of *Brucella* has progressed a great deal since the techniques of molecular biology have been applied to these bacteria. A number of molecular tools (nucleic probes, primers, etc.) are now available which make the elaboration of a more objective and reliable classification of the genus possible. Judging by the emergence of new *Brucella* types from marine mammals, the genus is far from being closed. In the near future, efforts should be concentrated on the harmonization of these tools to propose the most suitable method for the molecular identification and typing of *Brucella*.

3. EPIDEMIOLOGY AND CLINICAL ASPECTS

B. melitensis infection in sheep appears to occur naturally in the Mediterranean region, but the infection is widely spread.

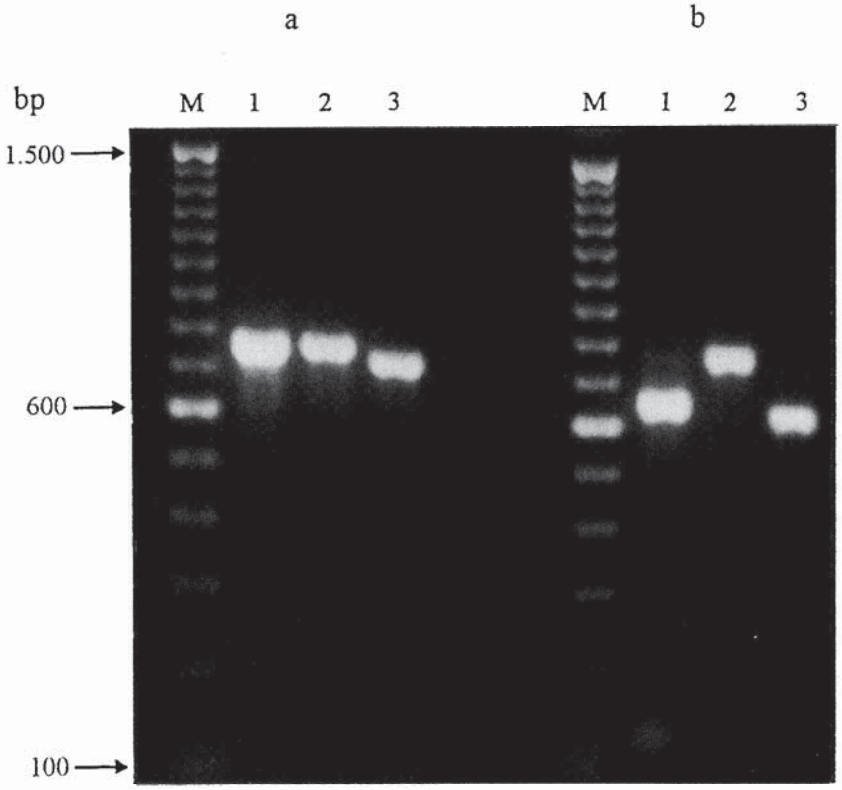

Figure 1. Electrophoretic patterns of the *omp25* gene amplified in *B. abortus* 544 (1), *B. melitensis* 16M (2) and *B. ovis* 63/290 (3), not cut (a) and cleaved by *Eco*RV (b). M: molecular mass marker (100 bp DNA ladder, Gibco).

However, North America is believed to be free, as are Northern Europe, Southeast Asia, Australia and New Zealand (FAO/OIE/WHO, 1997).

The main clinical manifestations of brucellosis in sheep are, as in all ruminants, reproductive failure, i.e. abortion and birth of weak offspring, in females, and orchitis and epididymitis in males. Arthritis is also observed occasionally.

B. melitensis infection of sheep is quite similar from both pathological and epidemiological standpoints to *B. abortus* infection in cattle. *B. melitensis* biovar 3 appears to be the most frequently isolated in Mediterranean countries. The precise recognition of biovar 3, especially its differentiation from biovar 2 appears sometimes equivocal. Due to the use of insufficiently discriminatory monospecific sera, a number of strains identified initially as biovar 2 were later confirmed as biovar 3 by expert laboratories. There is no evidence that either the epidemiological or clinical features of *B. melitensis* infection in sheep vary with the three different biovars (Fensterbank, 1987). In most circumstances, the primary dissemination way of *Brucella* is the placenta, fetal fluids and vaginal discharges expelled by

infected ewes after abortion or full-term parturition. Shedding of *Brucella* is also common in udder secretions and semen, and *Brucella* may be isolated from various tissues, such as lymph nodes from the head and those associated with reproduction, and from arthritic lesions (Alton et al., 1988). Similarly to *B. abortus* infection in cattle, *B. melitensis* can be transmitted from the ewes to lambs. A small proportion of lambs can be infected in utero, but the majority of *B. melitensis* infections are probably acquired through the colostrum or milk (Grilló et al., 1997). It is also probable that a self-cure mechanism similar to that suggested in cattle takes effect in most of the infected lambs (Grilló et al., 1997). Despite this low frequency of transmission, the existence of such latent infections would greatly increase the difficulty of eradicating this disease, as *B. melitensis* persists without having a detectable immune response. The exact mechanism of the development of latent *B. melitensis* infections remains unknown (Grilló et al., 1997).

4. DISEASE CONTROL

The strategies for the prevention and control of brucellosis in sheep are mainly based on the knowledge of the pathogenesis and epidemiology of the infection. General non-specific measures should be implemented, taking in account the long survival time of *B. melitensis* in the environment. Used exhaustively in whole-flock vaccination programmes, the live *B. melitensis* Rev 1 vaccine greatly decreases the prevalence of brucellosis in both sheep and human populations (Elberg, 1981, 1996). Once the prevalence has been diminished, a more efficient control of the disease may be achieved through the implementation of a programme based on the combination of Rev 1 vaccination of lambs with the test-and-slaughter of adults. Finally, it may be possible to use a test-and-slaughter programme only. This requires the exhaustive identification of animals and flocks and the control of animal movements. It also requires enough economic means to be implemented, but it could lead to the eradication of the disease (Garin-Bastuji, 1996).

When *B. melitensis* strain Rev 1 vaccine is administered by the standard method ($1–2 \times 10^9$ CFU injected subcutaneaously), it may induce a long-lasting serological response. In contrast, when this vaccine is administered by the conjunctival route, the immunity conferred is similar to that induced by the standard method but the serological response evoked is significantly reduced (Fensterbank et al., 1985). The classically recommended exclusive vaccination of young replacement animals has failed to control brucellosis in some developed countries and is frequently inapplicable in the developing world. As a result, whole-flock vaccination appears to be the only feasible alternative for controlling *B. melitensis* infection in small ruminants under the extensive management conditions of these countries. The vaccination of pregnant animals with full standard doses of Rev 1 administered subcutaneously or conjunctivally is followed by abortion in most vaccinated animals (Zundel et al., 1992; Blasco, 1997). Reducing the dose of vaccine has been suggested as a method of avoiding this problem and accordingly, a reduced dose vaccination has been widely used and has been reported as a safe and effective method of controlling small ruminant brucellosis (Elberg, 1981, 1996; Al Khalaf et al., 1992). However, field and experimental results support the fact that due to the induction of abortion in pregnant animals and the low degree of immunity conferred, reduced doses of Rev 1 should not be recommended as an alternative to the full standard doses (Zundel et al., 1992; Blasco, 1997).

When tested in a mouse model, differences in residual virulence and immunogenicity have been demonstrated between the different Rev 1 vaccines produced worldwide. The differences could account for the discrepancies in safety results obtained in mass vaccination trials in different countries (Blasco, 1997). The induction of abortions when vaccinating pregnant animals means that there is no entirely safe strategy for Rev 1 vaccination. Conjunctival vaccination is safer than subcutaneous vaccination but is not safe enough to be applied regardless of the pregnancy status of the ewes and should be used only under restricted conditions (Jiménez de Bagüés et al., 1989; Zundel et al., 1992). For sheep, conjunctival administration of standard doses of Rev 1 late in the lambing season or during lactation is recommended as a whole-flock vaccination strategy (Blasco, 1997).

New generation vaccines can be classified by the method by which they were obtained, by classical techniques, or mutagenesis or genetic engineering. Among the classically obtained *Brucella* strains with smooth LPS, there is *B. suis* S2 which was apparently successful in field experiments in China and Lybia (Mustafa and Abusowa, 1993), but showed no protection in controlled experiments against *B. melitensis* (Verger et al., 1995). *B. abortus* RB51, a rough stable strain, was protective against all *Brucella* species in a mouse model (Jiménez de Bagüés et al., 1994). The strain RB51 was also protective against *B. abortus* in cattle in USA without inducing levels of anti-O chain antibodies capable of being measured by serological tests (Palmer et al., 1997) but was not protective against *B. ovis* in controlled experiments in sheep (Jiménez de Bagüés et al., 1995). Up to now, there is no report on the efficacy of RB51 as a vaccine against *B. melitensis* in sheep. VRTM1 and VTRS1 are two live strains obtained by transposon mutagenesis from *B. melitensis* 16M and *B. suis* 2579, respectively. Both strains showed growth curves similar to those of the Rev 1 vaccine and were protective in the Balb/c model against *B. melitensis* biovar 1 (strain 16M) and *B. suis* biovars 1 (strain 750) and 4 (strain 2579) (Winter et al., 1996). Further studies are needed to characterize the immunity conferred by these new live strains against *B. melitensis* in small ruminants particularly.

5. DIAGNOSIS

5.1. Direct diagnosis

The most reliable and the only unequivocal method for diagnosing animal brucellosis is based on the isolation of *Brucella* spp. (Alton et al., 1988). The bacteriological diagnosis of *B. melitensis* can be made by means of the microscopic examination of smears from vaginal swabs, placentas or aborted fetuses stained with the Stamp modification of the Ziehl-Neelsen method. However, morphologically related microorganisms such as *B. ovis*, *Chlamydia psittaci* or *Coxiella burnetii* can cause misleading diagnoses. Therefore, the isolation of *B. melitensis* on appropriate culture media is recommended for an accurate diagnosis. Vaginal excretion of *B. melitensis* is usually copious and persists several weeks after abortion (Alton, 1990). Moreover, the mammary gland is the main target of infection in small ruminants (Marín et al., 1996a). Thus, taking vaginal swabs and milk samples is the best way to isolate *B. melitensis* from sheep. The spleen and lymph nodes (iliac, mammary and prefemoral) are the best areas for samples for isolation purposes in necropsied animals (Marín et al., 1996a).

B. melitensis does not require serum or CO_2 for growth and can be isolated on

ordinary solid media under aerobic conditions at 37 °C. However, the use of non-selective media cannot be recommended because of the overgrowing contaminants usually present in field samples, and selective media are needed for isolation purposes. The Farrell selective medium, developed for the isolation of *B. abortus* from milk (Farrell, 1974), is also recommended for the isolation of *B. melitensis* (Alton et al., 1988). However, nalidixic acid and bacitracin, at the concentration used in this medium, have inhibitory effects for some *B. melitensis* strains (Marín et al., 1996b). Thus, its sensitivity for the isolation of *B. melitensis* from naturally infected sheep is sometimes lower than that obtained with the less selective Thayer-Martin modified medium (Marín et al., 1996a). The sensitivity increases significantly by the simultaneous use of both the Farrell and the modified Thayer-Martin media (Marín et al., 1996b). Additional work should be carried out to develop a new selective medium that is more efficient and suitable for isolating all *Brucella* species.

While culturing is a specific method, its sensitivity depends on the viability of *Brucella* within the sample, the kind of sample (fetus organs, fetal membranes, lymph nodes, etc.) and the number of specimens tested from the same animal (Hornitzky and Searson, 1986). The time required for culturing field specimens can be long and tissues or fluids that are only contaminated with a low number of *Brucella* may not be detected. PCR assay has been shown to be a valuable method for detecting DNA from different microorganisms and provides a promising option for the diagnosis of brucellosis. Several authors reported good sensitivity of PCR for detecting of *Brucella* DNA with pure cultures (Fekete et al., 1990a, b; Baily et al., 1992; Herman and De Ridder, 1992; Romero et al., 1995a; Da Costa et al., 1996). Others showed that PCR could be a potentially useful tool when used alone (PCR, AP-PCR, rep-PCR, ERIC-PCR) or in combination with labelled probes to differentiate some *Brucella* species and biovars (Fekete et al., 1992b; Bricker and Halling, 1994, 1995; Cloeckaert et al., 1995; Mercier et al., 1996; Ouahrani-Bettache et al., 1996; Tcherneva et al., 1996). However few studies have been performed with clinical or field samples (Fekete et al., 1992a; Leal-Klevezas et al., 1995; Romero et al., 1995b; Matar et al., 1996; Rijpens et al., 1996). The possibility of using the PCR technique to detect the DNA of dead bacteria, or in paucibacillary samples and even in samples highly contaminated with other microorganisms, could increase the rate of detecting animals infected by *Brucella*. However, up to now, no technique is sensitive enough to replace classical bacteriology on all kinds of biological samples.

5.2. Indirect diagnosis

5.2.1. Immune response

As mentioned before, the *B. melitensis* Rev 1 strain is the best vaccine available, but when applied under standard conditions (i.e. full dose via the subcutaneous route in young replacement animals) it induces long lasting serological responses that seriously interfere with subsequent serological screening (Alton and Elberg, 1967; Elberg, 1981, 1996; Alton, 1990; MacMillan, 1990). As no differences have been found between the diagnostic antigens, those from field strains of *B. melitensis* and those from the Rev 1 vaccine, it is difficult to find a serological test able to distinguish infected from vaccinated animals. This problem currently impedes the combined use of vaccination and test and slaughter programmes for eradicating brucellosis.

5.2.2. Diagnostic antigens

There is no scientific agreement on what should be the nature and characteristics of a universal antigen for diagnosing brucellosis due to smooth *Brucella* (*B. abortus*, *B. melitensis* and *B. suis*). One of the most critical and controversial points concerning the serological diagnosis of *B. melitensis* infection in small ruminants is related to which *Brucella* species and biovars are used in the production of the diagnostic antigens. The rose bengal test (RB) and the complement fixation test (CF) are the most widely used tests for the serological diagnosis of sheep brucellosis (Farina, 1985; MacMillan, 1990), and are currently the official tests used in the European Union countries (European Council Directive 64/432/EEC, 1964). The antigenic suspensions (whole cells) used in both tests are made with *B. abortus* biovar 1 (an A-dominant strain) (Alton et al., 1988) which means that, theoretically, infections due to M-dominant strains (*B. melitensis* biovar 1; *B. abortus* biovars 4, 5 and 9; *B. suis* biovar 5) could be misdiagnosed (Alton et al., 1988; MacMillan, 1990). However, recent results showed no significant difference in the sensitivity of the classical RB antigen prepared with *B. abortus* biovar 1 (A-dominant) between ovine populations infected with either biovar 1 (M-dominant) or biovar 3 (A-dominant) of *B. melitensis* (Blasco et al., 1994b).

The outer membrane of the bacteria contains the main antigens involved in the humoral response against *Brucella* (Díaz et al., 1968a). As in other Gram-negative bacteria, the outer membrane of smooth *Brucella* is composed of phospholipids, proteins and lipopolysaccharide (smooth lipopolysaccharide, S-LPS). The S-LPS is the immunodominant antigen and most of serological tests, particularly those using whole-cell suspensions as antigen (such as RB, CF), and most ELISA tests, have been developed to detect antibodies to this antigen (Díaz et al., 1968a). The S-LPS of smooth *Brucella* is composed of an inner glycolipidic moiety (the core oligosaccharide plus the lipid A) and an outer polysaccharide chain (O-chain). This O-chain is the relevant antigenic moiety and is chemically composed of a perosamin homopolymer showing α-1,2 and α-1,3 linkages (Cherwonogrodzky et al., 1990). The O-chain polysaccharide of *B. abortus* biovar 1 (A-dominant) possesses a fine structure with only a low-frequency (ca 2 %) of α-1,3 linked 4,6-dideoxy-4-formamido-D-mannopyranose residues. In contrast, the O-chain polysaccharide of *B. melitensis* biovar 1 (M-dominant) contains repeated pentasaccharide units with one α-1,3 and four α-1,2 linkages. As a result, the A and M antigenic characteristics depend on the O polysaccharides in which the frequency of α-1,3 linked residues varies. Studies with monoclonal antibodies (Douglas and Palmer, 1988) show that the A epitope is related to portions of at least five sugars with α-1,2 linkages and that the M epitope includes sugars with α-1,3 linkages (thus its relevance in the O-chain of *B. abortus* biovar 1 should not be important). Therefore, all biovars assigned as A-dominant should express few or no α-1,3 linked residues, while M-dominant strains possess a unique M epitope as well as a di-, tri- or tetrasaccharides with α-1,2 linkages, and can thus be considered to be contained within the A epitope structure (Bundle et al., 1989; Meikle et al., 1989; Cherwonogrodzky et al., 1990). The presence of common oligosaccharides of four or less sugars is consistent with the existence of a common (C) epitope. Indeed, this C epitope has been detected with the appropriate monoclonal antibodies (Douglas and Palmer, 1988) and can account for the high sensitivity of the antigens made from A-dominant strains (i.e. *B. abortus* biovar 1) at detecting M-dominant *B. melitensis* biovar 1 infections and vice-versa (MacMil-

lan, 1990; Díaz-Aparicio et al., 1993). In fact, crude LPS extracts from either *B. melitensis* 16M (biovar 1, M-dominant) or *B. abortus* 2308 (biovar 1, A-dominant) are equally sensitive in an indirect ELISA (i-ELISA) for diagnosing brucellosis in sheep infected by *B. melitensis* biovar 1 (Marín et al., unpublished results). However, the native hapten and the S-LPS hydrolytic polysaccharides containing the O-chain and core sugars from *B. abortus* biovar 1 fail to react in precipitation tests with a large proportion of *B. melitensis* infected sheep, goats and cattle under conditions in which the same antigens obtained from *B. melitensis* biovar 1 detected most of those animals (Díaz-Aparicio et al., 1993). Therefore, further research is needed to clarify the practical importance and interest of using species-specific diagnostic antigens for the different serological tests.

There is limited information on the value of outer membrane and inner cytoplasmic proteins for the diagnosis of *B. melitensis* infection in sheep.

The immunoelectrophoretical patterns of cytoplasmic proteins show little differences between *Brucella* species when assayed with polyclonal sera (Díaz et al., 1967, 1968b). These inner antigens are considered specific for the genus, being useful to differentiate infections due to *Brucella* from those due to bacteria whose LPS cross-reacts with the *Brucella* S-LPS, as is the case of *Yersinia enterocolitica* O:9 (Díaz and Bosseray, 1974). However, a cross-reactivity among cytosolic proteins of *B. melitensis* and those obtained from *Ochrobactrum anthropi*, an opportunist human pathogen, has been reported recently (Velasco et al., 1997). The *Brucella* cytoplasmic antigens, known also as brucellin (Jones et al., 1973) have been used successfully for the allergic diagnosis of brucellosis in sheep and goats (Fensterbank, 1982, 1985; Ebadi and Zowghi, 1983; Loquerie and Durand, 1984; Blasco et al., 1994b). Moreover, these cytoplasmic antigens have been reported to be sensitive and specific enough for the diagnosis of brucellosis in sheep and goats when used in precipitation tests (Muhammed et al., 1980; Trap and Gaumont 1982; Díaz-Aparicio et al., 1994). In contrast, when these cytoplasmic antigens are used in the i-ELISA, the sensitivity obtained is not adequate due to the high background IgG reactivities with sera from *Brucella*-free animals (Díaz-Aparicio et al., 1994; Salih-Alj Debbarh et al., 1996). An important drawback of diagnostic tests using uncharacterized cytosolic proteins is the lack of specificity when testing Rev 1 vaccinated sheep and goats. But a partially purified cytosoluble protein of 28 kDa (CP28) from the cytosoluble protein extract (CPE) of *B. melitensis* has been reported as being able to differentiate Rev 1 vaccinated from *B. melitensis* infected ewes when used in i-ELISA (Debbarh et al., 1995). However this test is less sensitive than both the RB and CF tests for diagnosing *B. melitensis* infected ewes (Salih-Alj Debbarh et al., 1996). The corresponding *B. melitensis* 16M *bp26* gene was expressed in *Escherichia coli* and monoclonal antibodies were produced (Cloeckaert et al., 1996a, b). Sequence analysis of the cloned gene revealed that it was nearly identical to the recently published *B. abortus bp26* gene, coding for a periplasmic protein (Rossetti et al., 1996). A competitive ELISA (c-ELISA) using CPE as antigen and some of these monoclonal antibodies improved the sensitivity for diagnosing infected sheep, and no antibody response was detected in Rev 1 vaccinated sheep (Debbarh et al., 1996).

Several authors have attempted to identify the main polypeptide specificities of the antibody response to outer-membrane protein (OMP) extracts of *B. melitensis* by using either immunoblotting or c-ELISAs with specific monoclonal antibodies (Zygmunt et al., 1994a, b; Debbarh

et al., 1995; Hemmen et al., 1995; Tibor et al., 1996). While OMPs of 10, 17, 19, 25–27 and 31–34 kDa were found that were suitable as potential antigens for the diagnosis of brucellosis in sheep by immunoblotting or ELISA, the antibody response to OMP was very low and heterogeneous in *B. melitensis* infected sheep (Zygmunt et al., 1994a, b).

Further research is needed on the identification, isolation, characterization and cloning of both inner and outer membrane proteins which could be used as diagnostic antigens that are more sensitive and specific. This should be followed by the development of subunit or live antigen-deleted vaccines, able to protect animals without interfering with diagnostic tests, and should be a major goal of research in the near future.

5.2.3. Serological tests

No specific serological tests for *B. melitensis* infection of sheep have been developed and it is widely assumed that the serological tests used for *B. abortus* infection in cattle are also adequate for the diagnosis of *B. melitensis* infection in small ruminants. Accordingly, the RB and CF are the most widely used tests for the serological diagnosis of brucellosis in sheep and goats (Farina, 1985; Alton, 1990; MacMillan, 1990).

The RB test was developed more than 20 years ago for the diagnosis of bovine brucellosis and, despite the scanty and sometimes conflicting information available (Trap and Gaumont, 1975; Fensterbank and Maquere, 1978; Farina, 1985; MacMillan, 1990; Alton, 1990; Blasco et al., 1994a, b), this test is internationally recommended for the screening of brucellosis in small ruminants (Joint FAO/WHO expert committee on Brucellosis, 1986; Garin-Bastuji and Blasco, 1997). An important problem affecting the sensitivity of the RB test concerns the standardization of the antigens. The European Union regulations require antigen suspensions in lactate buffer at pH 3.65 ± 0.05 that are able to agglutinate at a dilution of 1:47.5 (21 IU/mL) of the International Standard anti-*B. abortus* serum (ISaBS) but which give a negative reaction at a dilution of 1:55 (18.2 IU/mL) of the same serum (European Council Directive, 1964). These standardization conditions, which seem to be suitable for the diagnosis of *B. abortus* infection in cattle (MacMillan, 1990), are not adequate for the diagnosis of *B. melitensis* infection in sheep (Blasco et al., 1994a, b). This accounts for the relatively low sensitivity of some commercial RB antigens at diagnosing brucellosis in sheep and goats (Falade, 1978, 1983; Blasco et al., 1994a) and for the fact that a high proportion of sheep and goats belonging to *B. melitensis*-infected areas give negative results in the RB but positive ones in the CF (Blasco et al., 1994a). These phenomena seriously question the efficacy of using the RB as an individual test in small ruminants. At least for sheep, the sensitivity of the RB test improves significantly when the antigens are standardized against a panel of sera from several *B. melitensis* culture positive and *Brucella*-free sheep (Blasco et al., 1994a).

The CF is the most widely used test for the serological confirmation of brucellosis in animals. As in cattle brucellosis, despite its complexity and the heterogeneity of the techniques used in the different countries, there is agreement that this test is effective for the serological diagnosis of brucellosis in sheep and goats (Farina, 1985; MacMillan, 1990; Alton, 1990). When testing a limited number of sera obtained from *B. melitensis* culture positive and *Brucella*-free goats, the CF test provided the same sensitivity as those of the RB and i-ELISA (Díaz-Aparicio et al., 1994). However, under field conditions, the sensitivity of the CF test has been

reported to be somewhat lower (88.6 %) than those of the RB (92.1 %) and i-ELISA (100 %) for diagnosing *B. melitensis* infection in sheep (Blasco et al., 1994a, b). On the other hand, the CF test has many drawbacks such as complexity, variability of reagents, prozones, anticomplementary activity of sera, difficulty to perform with hemolysed sera, and subjectivity of the interpretation of low titers. Therefore, while the sensitivity of RB is sufficient for the surveillance of free areas at the flock level, RB and CF should be used together in infected flocks to obtain accurate individual sensitivity in test-and-slaughter programmes. Moreover, an important drawback of both RB and CF tests is their low specificity when testing sera from sheep and goats vaccinated subcutaneously with Rev 1 (Fensterbank et al., 1982; Jiménez de Bagüés et al., 1992; Díaz-Aparicio et al., 1994). However, when the Rev 1 vaccine is applied conjunctivally (Fensterbank et al., 1982), the interference problem is significantly reduced in all serological tests (Jiménez de Bagüés et al., 1992; Díaz-Aparicio et al., 1994).

Relatively little information is available on the value of the ELISA for the diagnosis of *B. melitensis* in small ruminants. The indirect ELISA, using more or less purified S-LPS of *B. melitensis* as antigen and polyclonal conjugate (anti-IgG H+L), has been reported to be sensitive enough for the diagnosis of infection in sheep and goats (Jiménez de Bagüés et al., 1992; Díaz-Aparicio et al., 1994; Blasco et al., 1994b; Delgado et al., 1995). A similar technique has been also proposed for diagnosing sheep brucellosis in individual or pooled milk samples (Biancifiori et al., 1996), but due to the low rate and frequency of *Brucella* antibodies in milk, the test lacks sensitivity, compared with tests performed on serum. One of the problems of the i-ELISAs performed on serum is the high background reactivity obtained when testing sera from *Brucella* free animals (Jiménez de Bagüés et al., 1992). The use of protein G as conjugate significantly reduces this problem, increasing the ELISA specificity (Díaz-Aparicio et al., 1994; Ficapal et al., 1995). This increased specificity is also obtained when testing *Brucella*-free sheep in the above i-ELISA but using a monoclonal anti-ruminant IgG1 conjugate. However, the sensitivity of i-ELISAs with either protein G or monoclonal conjugates decreases with respect to that obtained with the polyclonal conjugate (Blasco et al., unpublished results). Literature references available on the use of competitive ELISA (c-ELISA) protocols for the diagnosis of brucellosis in sheep are scanty. In our experience, competitive protocols using an anti-C epitope monoclonal antibody did not outperform conventional i-ELISAs for the diagnosis of *B. melitensis* in sheep and goats (Moreno et al., unpublished results). As happens with the other serological tests, the specificity of the ELISAs is quite low when testing sera from Rev 1 vaccinated animals (Jiménez de Bagüés et al., 1992; Díaz-Aparicio et al., 1994; Moreno et al., unpublished results). However, as mentioned before, the use of a purified periplasmic protein (26 kDa) in i-ELISA or c-ELISA protocols could be useful for differentiating *B. melitensis* infections from Rev 1 vaccinated sheep (Debbarh et al., 1996; Salih-Alj Debbarh et al., 1996).

Further research is needed to develop serological tests of improved sensitivity for the diagnosis of brucellosis in sheep, which would be able to discriminate between infected and vaccinated animals.

5.2.4. Cell mediated immunity (CMI) based diagnosis

The detection of the delayed-type hypersensitivity (DTH) reaction (skin test) has been used for the diagnosis of sheep

brucellosis with variable success (Jones and Marly, 1975; Fensterbank, 1982, 1985; Loquerie and Durand, 1984; Blasco et al., 1994b). The allergens used in early studies were generally obtained from culture supernatants or by acid extraction of smooth *Brucella* cells (Alton, 1990) and, therefore, contained S-LPS or its hydrolytic polysaccharides. Since the S-LPS does not take part in DTH reactions (Jones et al., 1973; Jones, 1974) and, in contrast, its O-chain elicits a strong antibody response, injection of minimal amounts of S-LPS in previously sensitized animals could cause an inflammatory reaction interfering with the interpretation of the skin test. Moreover, such extracts could cause a secondary antibody response interfering with future serological testing. These problems are practically solved by using strains devoid of the O-chain polysaccharide, as is the case of the rough *B. melitensis* 115 (Jones et al., 1973). The allergens can be obtained from this strain by several methods (Bhonghibhat et al., 1970; Jones et al., 1973; Dubray, 1985; Blasco et al., 1994b). Despite the existence of quantitative and qualitative differences among the allergens obtained by these different methods, the results show that purified allergens would not offer practical advantages in sensitivity and specificity over complex protein mixtures (Blasco et al., 1994b). The site and route of allergen inoculation are not important factors affecting the sensitivity of skin test for brucellosis (Fensterbank, 1985; Alton, 1990; Blasco et al., 1994b). The method considered more efficient and practical for sheep is the subcutaneous inoculation in the lower eyelid with readings 48 h after inoculation (Jones et al., 1973; Jones and Marly, 1975; Fensterbank, 1985). However, since mixed DTH-antibody mediated intradermal reactions are occasionally observed, a reading time of 72 h seems advisable for a better assessment of true DTH reactions (Blasco et al., 1994b). Anergy induced by repeated skin testing is a well known phenomenon in bovine tuberculosis (Radunz and Lepper, 1985). This phenomenon is not absolute in the case of brucellosis in sheep, but the skin test responses to *Brucella* allergens lessen within the 24 days that follow a positive skin test (Blasco et al., 1994b). The skin test is considered as always being negative when testing unvaccinated *Brucella*-free animals (Fensterbank, 1982, 1985; Loquerie and Durand, 1984; Blasco et al., 1994b). In contrast, the skin test is positive in many Rev 1 vaccinated animals (2 years or more after vaccination), thus lacking specificity for differentiating infected from vaccinated sheep (Fensterbank, 1982; Pardon et al., 1989). No information has been published on the diagnostic value of in vitro CMI tests (i.e. blastogenesis, IL and IFNγ detection, etc.) for the diagnosis of brucellosis in small ruminants.

6. CONCLUSION

Clearly knowledge concerning *B. melitensis* infection in sheep has dramatically progressed within the past 20 years. Even though many aspects require additional research, several diagnostic and prophylactic tools have been sufficiently validated and standardized, and are readily available to control the disease efficiently.

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