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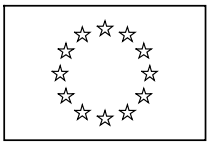

EUROPEAN COMMISSION

HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Health Opinions

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Brucellosis in Sheep and Goats

(*Brucella melitensis*)

Scientific Committee on Animal Health and Animal Welfare

Adopted 12 July 2001

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Report of the

Scientific Committee on Animal Health and Animal Welfare

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1. EXECUTIVE SUMMARY

Tests currently used for the serological diagnosis of *B. melitensis* infections in sheep and goats were initially developed for the diagnosis of *B. abortus* infections in cattle. Although not formally validated for use in sheep and goats, these tests, and in particular the Rose Bengal plate agglutination test, the complement fixation test, and more recently the ELISA have been used for the serological diagnosis of brucellosis in sheep and goats. A combination of tests shows a degree of sensitivity and specificity which appears sufficient to detect infected animals, and removal of those animals appears to contribute to disease control when vaccination is avoided or when the Rev.1 vaccine is only administered in young animals, particularly by the conjunctival route. In other situations, especially when the vaccine is administered in adult animals, the results of these tests can sometimes be difficult to interpret because of antibodies induced by vaccination. It is recommended that the existing diagnostic tests are improved and validated. Direct proof of *Brucella* infection requires isolation of bacteria with well established methods or detection of bacterial genome by application of polymerase chain reaction (PCR).

The Rev.1 live *B. melitensis* vaccine is the most widely used vaccine in control programmes against brucellosis in small ruminants. When properly used the Rev.1 vaccine confers a long lasting protection against field infections in a high proportion of animals. This vaccine however shows a considerable degree of virulence and induces abortions when the first vaccine dose is administered during pregnancy. The antibody response to vaccination cannot be differentiated from the one observed after field infection, and this therefore impedes control programmes. Attempts have been made to develop new vaccines based on “rough” (R) strains or genetically modified strains of the Brucella species. Those vaccines await further evaluation in field experiments.

Control programmes in place include the proper application of vaccination as an indispensable step on the way to eradication of *B. melitensis* in the field. Any control programme requires a well functioning surveillance system, the co-operation with livestock owners and considerable financial support. The appropriate application of vaccination will in any case result in a suppression of the infection pressure and has been shown to reduce the zoonotic spread of the disease.

2. REQUEST FOR OPINION AND INTRODUCTION

The Scientific Committee on Animal Health and Animal Welfare is asked to:

1. Comment on the sensitivity, specificity and practical usefulness of the various diagnostic tests for *Brucella melitensis* in sheep and in goats.

2. Comment on the available vaccines, the level of protection resulting, and the effects of vaccine use on the above tests.

3. Comment on the role of eradication programmes and vaccine use in reducing the zoonotic spread of the disease.

This report presents a general review of the subject and specifically addresses the above questions as follows. The diagnostic tests for *B. melitensis* are reviewed in Chapter 8 and vaccines and their impact on diagnostic tests are covered in Chapter 9. The selection of eradication strategies and their impact on the prevalence of the disease in man is covered in Chapter 10. A summary of the various conclusions of the Committee is presented in Chapter 12.

3. HISTORY AND IMPORTANCE

Sheep and goats brucellosis (excluding *Brucella ovis* infection which is not pathogenic for humans) is a zoonotic infection with important effects on both public health and animal health and production and is widespread in many areas of the world, particularly in some Mediterranean and Middle Eastern countries.

Brucella melitensis, the main aetiologic agent of brucellosis in small ruminants, was the first species in the genus *Brucella* described. It was first isolated by Bruce in 1887 (Alton, 1990) from the spleens of soldiers dying of Mediterranean fever on the island of Malta. Bruce called it *Micrococcus melitensis*. The origin of the disease remained a mystery for nearly 20 years until it was discovered that goats were the source of infection for human populations.

4. ZOONOTIC ASPECTS OF *BRUCELLA MELITENSIS* INFECTION

Human brucellosis is widely distributed all over the world, with regions of high endemicity such Mediterranean, Middle East, Latin America and parts of Asia (Corbel, 1997, López-Merino, 1989). The true incidence of human brucellosis is unknown. Reported incidence in endemic-disease areas varies widely, from <0.01 to >200 per 100,000 population (López-Merino, 1989).

Humans are accidentally infected and almost always dead-end hosts of *Brucella* infections. The disease is primarily an occupational risk in exposed professions, *i.e.*, veterinarians, farmers, laboratory technicians, abattoir workers, and others who work with animals and their products. People living near infected premises may also contract infection. The primary source is the animal and infection is contracted either by direct or indirect contact through the skin or mucous membranes or ingestion of contaminated products, especially fresh dairy products. The maximum danger is therefore during the lambing or kidding period. Dairy products are the

main source of infection for people who do not have direct contact with animals. Much of the milk which is consumed is now rendered safe by pasteurisation or boiling, but cheese made from sheep and goat milk is preferably prepared from untreated milk and by the use of rennet from lambs and kids that may have come from *Brucella* infected animals. During the course of cheese manufacture, any *Brucella* present in the milk become trapped in the clot and thus concentrated in the cheese, although bacteria may subsequently be inactivated by manufacturing or ripening processes. Cream and ice cream prepared from goat milk has also been incriminated (Flores-Castro and Baer, 1980). The prevalence of human brucellosis acquired from dairy products is seasonal, reaching a peak soon after kidding and lambing. Abattoir workers handling infected animals are also at risk, especially from the contents of uteri and udders. The handling of raw wool has been identified as a potential source of infection of workers involved. Finally, *B. melitensis* is easily acquired by laboratory infection.

Humans are susceptible to *B. abortus*, *B. melitensis* and *B. suis*. *B. melitensis* and *B. suis* often give rise to a severe and long lasting form of the disease. After an incubation period of 8 to 20 days, illness occurs in different forms. Asymptomatic infection is frequent and mainly due to *B. abortus*. It is characterised by antibody formation in persons with no history of symptoms consistent with brucellosis. The acute form of the disease is also common and symptoms include lassitude, headache and muscular or joint pain, and drenching sweats, especially at night. The manifestations of brucellosis are sometimes more pronounced or limited to a specific system or organ. This is then termed a *complication* when it occurs in the course of acute infection, or *localised brucellosis* when occurring in the absence of other signs of systemic illness. The most common localisations are spondylitis, peripheral arthritis, especially of the hip, knee and shoulder, or epididymo-orchitis. Nervous, genitourinary, hepatosplenic and cardiovascular complications may also be observed. Brucellosis is termed *chronic* when it includes one or more of the signs described above and persists or recurs over a period of six months or more. Finally, *Brucella* dermatitis has traditionally been ascribed to "allergy" to *Brucella*.

The disease presents a great variety of clinical manifestations, making it difficult to diagnose clinically. Therefore, the diagnosis must be confirmed directly by isolation of *Brucella*, mostly from blood culture, or indirectly by the detection of immune response against its antigens. The diagnosis of brucellosis based exclusively upon *Brucella* isolation presents several drawbacks (Orduña *et al.,* 2000). The slow growth of *Brucella* in primary cultures means that diagnosis may take more than 7 days (Ariza, 1996, Rodríguez-Torres *et al*., 1987, Yagupsky, 1999). Besides, blood culture sensitivity is often low, ranging from 50-90 % depending on disease stage, *Brucella* species, culture medium, number of circulating bacteria and the culture technique employed (Gotuzzo *et al.,* 1986, Yagupsky *et al*., 1999). Hence, serological tests play a major role in diagnosis when the agent cannot be detected by blood culture. Yet, the interpretation of these tests is often difficult, particularly in patients with chronic brucellosis, in re-infections and relapses, and in endemic areas where a high portion of the population carries antibodies against brucellosis (Orduña *et al.,* 2000).

Various serological tests have been used for the diagnosis of human brucellosis. The most common tests used are serum agglutination test (SAT), Coombs anti-*Brucella* test, Rose Bengal test and complement fixation test (Orduña *et al*., 2000). During

the last decade, radioimmunoassays (Hewitt and Payne, 1984, Parrat *et al*., 1977) and in particular enzyme-immunoassays (Ariza *et al*., 1992, Gazapo *et al*., 1989, Saz *et al*., 1987) have also been used.

Other tests have proved useful in some patients, such as the indirect immunofluorescence test, Brucellin counter-electrophoresis and passive haemagglutination test, but their value in clinical practice is still under assessment. Allergic tests reveal a delayed-type hypersensitivity; using conventional antigen preparations. Brucellin-INRA, a S-LPS free product was reported as reliable and innocuous, but further work is necessary.

Treatment of choice in acute brucellosis consists of antibiotic therapy. The best results are achieved with rifampicine combined with doxycycline for at least 6 weeks. Treatment generally needs to be prolonged or repeated in persistent forms before a cure is achieved.

4.1. Conclusions

Human brucellosis is distributed worldwide, with regions of high endemicity such as the Mediterranean region, Middle East, Latin America and parts of Asia. The true incidence of human brucellosis is unknown. Reported incidence in endemic-disease areas varies widely, from <0.01 to >200 per 100,000 population. Humans are susceptible to different species of Brucella. Symptoms differ considerably, making clinical diagnosis difficult. Isolation of bacteria from blood samples has a sensitivity ranging from 50 – 90 %. Serology plays a major role, but interpretation of results is often inconclusive.

5. DESCRIPTION OF THE CAUSATIVE AGENT

Brucellosis in sheep and goats is primarily caused by *Brucella melitensis*, and rarely by *B. abortus* (Luchsinger and Anderson, 1979; Garin-Bastuji *et al.*, 1994) or *B. suis* (Paolicchi *et al.*, 1993).

5.1. Morphology

Brucella are coccobacilli or short rods 0.6 to 1.5 µm long by 0.5 to 0.7 µm in width. They are arranged singly and less frequently in pairs or small groups. The morphology of Brucella is fairly constant except in old cultures, where pleomorphic forms may be evident. Brucella are non-motile. They do not form spores, flagella, or pili. True capsules are not produced. Brucella are Gram-negative and usually do not show bipolar staining. They are not truly acid-fast but resist decolouration by weak acids, thus stain red by the Stamp's modification of Ziehl-Neelsen method, which is sometimes used for the microscopic diagnosis of brucellosis from smears of solid or liquid specimens (See Table 1 for differential characteristics).

Table 1: Differential characteristics of *Brucella* from some other Gram negative bacteria (Alton *et al.*, 1988)

Tests	**Brucella**	**Bordetella bronchiseptica**	**Campylobacter fetus**	**Moraxella**	**Acinetobacter**	**Yersinia enterocolitica O:9**
Morphology	small coccobacilli	small coccobacilli	comma	diplococcoid	diplococcoid	rod
Motility at 37°C	-	+	+	-	-	-
Motility at 20°C	-	-	-	-	-	+
Lactose fermentation on Mac Conkey agar	-	-	-	V[a]	V	-
Acid production on agar containing glucose	-[b]	-	-	-	V	+
Haemolysis on blood agar	-	+	-	V	V	-
Catalase	+	+	+	V	+	+
Oxidase	+[c]	+	+	+	-	-
Urease	+[d]	+	-	V	V	+
Nitrate reduction	+[e]	+	+	V	-	+
Citrate utilization	-	+	-	-	V	-
Agglutination with:S-Brucella antiserum	+[f]	-	-	-	-	+
R-Brucella antiserum	+[g]	-	-	-	-	-

a : Positive and negative species within the genus

b B. neotomae may show some fermentation

c :Except B. ovis, B. neotomae and some strains of B. abortus

d Except B. ovis and some strains of B. abortus

e Except B. ovis

f Except B. ovis , B. canis and R-forms of other species

g B. ovis , B. canis and R-forms of other species

5.2. Culture and growth characteristics

Brucella members are aerobic, but some strains require an atmosphere containing 5-10% carbon dioxide (CO_2) added for growth, especially on primary isolation. The optimum pH for growth varies from 6.6 to 7.4, and culture media should be adequately buffered near pH 6.8 for optimum growth. The optimum growth temperature is 36-38°C, but most strains can grow between 20°C and 40°C.

Brucellae require biotin, thiamin and nicotinamide. The growth is improved by serum or blood, but haemin (V-factor) and nicotinamide-adenine dinucleotide (X-factor) are not required. The growth of most *Brucella* strains is inhibited on media containing bile salts, tellurite or selenite.

Growth is usually poor in liquid media unless culture is vigorously agitated. Growth in static liquid media favours dissociation of smooth-phase cultures to non-smooth forms. Continuous and vigorous aeration will prevent this, provided a neutral pH is maintained. In semisolid media, CO_2-independent *Brucella* strains produce a uniform turbidity from the surface down to a depth of a few millimetres, while cultures of CO_2-requiring strains produce a disk of growth a few millimetres below the surface of the medium.

On suitable solid media *Brucella* colonies are visible after 2 days incubation. After 4 days incubation, *Brucella* colonies are round, 1-2 mm in diameter, with smooth (S) margins, translucent and a pale honey colour when plates are viewed in the daylight through a transparent medium. When viewed from above, colonies appear convex and pearly white. Later, colonies become larger and slightly darker.

Smooth *Brucella* cultures, especially *B. melitensis* cultures, have a tendency to undergo variation during growth, especially with subcultures, and dissociate to rough (R) forms, and sometimes mucoïd (M) forms. Colonies are then much less transparent with a more granular, dull surface (R) or a sticky glutinous texture (M), and range in colour from matt white to brown in reflected or transmitted light. Intermediate (I) forms between S, R and M forms may occur in cultures undergoing dissociation to the non-smooth state. Changes in the colonial morphology are generally associated with changes in virulence, serological properties and phage sensitivity.

5.3. Biochemistry

The metabolism of *Brucella* is oxidative and *Brucella* cultures show no ability to acidify carbohydrate media in conventional tests. The *Brucella* species are catalase positive and usually oxidase positive, and they reduce nitrate to nitrite (except *B. ovis* and some *B. canis* strains). The production of H_2S from sulphur containing amino-acids also varies. *B. melitensis* does not produce H_2S. Urease activity varies from fast to very slow. Indole is not produced from tryptophane and acetylmethycarbinol is not produced from glucose.

5.4. Antigenic characteristics

All smooth *Brucella* strains show complete cross-reaction with each other in agglutination tests with unabsorbed polyclonal antisera, a cross-reaction which does not extend to non-smooth variants. Cross-reactions between non-smooth strains can be demonstrated by agglutination tests with unabsorbed anti-R sera. Lipopolysaccharide (LPS) comprise the major surface antigens of the corresponding colonial phase involved in agglutination. The S-LPS molecules carry the A and M antigens, which have different quantitative distribution among the smooth *Brucella* strains. This is of value in differentiating biovars of the major species using absorbed monospecific A and M antisera.

Serological cross-reactions have been reported between smooth *Brucella* and various other Gram negative bacteria, *e.g. Escherichia coli* O:116 and O:157, *Salmonella* group N(O:30) of Kaufmann-White, *Pseudomonas multophilia*, *Vibrio cholerae* and especially *Yersinia enterocolitica* O:9. These organisms can induce significant levels of antibodies which cross-react with S-LPS *Brucella* antigens in diagnostic tests.

5.5. Susceptibility to phages

Over 40 *Brucella* phages have been reported to be lytic for *Brucella* members. All phages are specific for the genus *Brucella*, and are not known to be active against any other bacteria that have been tested. Thus, lysis by *Brucella* phages is a useful test to confirm the identity of *Brucella* spp. and for speciation within the genus. The *Brucella* phages currently used for *Brucella* typing are: Tbilisi (Tb), Weybridge (Wb), Izatnagar$_1$ (Iz$_1$) and R/C. The three former phages are used for differentiation of smooth *Brucella* species. R/C is lytic for *B. ovis* and *B. canis* (see Table 2).

Table 2: Differential characteristics of *Brucella* phages

	Lysis by phages (1)		
Species	Tb	Iz	R/C
B. melitensis	-	+	-
B. ovis	-	-	+
B. abortus	+	+	-

(1) At the routine test diluition

5.6. Susceptibility to dyes and antibiotics

Susceptibility to the dyes, thionin and basic fuchsin (20 µg/ml), which varies between biovars, is one of the routine typing tests of *Brucella*. *B. melitensis* grows in the presence of both dyes.

On primary isolation, *Brucellae* are usually susceptible *in vitro* to gentamicin, tetracyclines and rifampicine. Most strains are also susceptible

to the following antibiotics: ampicillin, chloramphenicol, cotrimoxazole, erythromycin, kanamycin, novobiocin, spectinomycin and streptomycin, but variation in susceptibility may occur between species, biovars and strains. Most strains are resistant to ß-lactamins, cephalosporins, polymyxin, nalidixic acid, amphotericin B, bacitracin, cycloheximide, clindamycin, lincomycin, nystatin and vancomycin at therapeutic concentrations.

Penicillin is used for the routine differentiation of the vaccinal strain *B. abortus* species biovar 1 strain 19, and streptomycin for *B. melitensis* biovar 1 strain Rev.1, the vaccines widely used for immunisation of cattle and small ruminants, respectively, from the virulent field strains of their respective biovars by virtue of their different sensitivity to these antibiotics (Alton *et al.,* 1988).

5.7. Taxonomy of *Brucella* species and biovars

Considering their high degree of DNA homology (> 90 % for all species), *Brucellae* 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 4 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...). 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 hydrogen sulphide (H_2S), dye (thionin and basic fuchsin) sensitivity, and agglutination with monospecific A and M anti-sera (see Table 3). Moreover, a recently developed co-agglutination test, using latex beads coated with a pair of monoclonal antibodies 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 accurately differentiate *B. ovis* from *B. canis* and the occasional rough isolates of the smooth *Brucella* species. *B. melitensis* biovar 3 appears to be the most frequently biovar 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 discriminating monospecific sera, a number of strains identified initially as biovar 2 were later confirmed as biovar 3 by expert laboratories.

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.

Table 3: Biovar differentiation of *Brucella* species involved in sheep and goat brucellosis

Species	Biovar or colonial morphology	CO_2 requirement	H_2S production	Growth on dyes (1)		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	+(2)	+	-	+	+	-	-	-
	2	+(2)	+	-	-	+	-	-	-
	3	+(2)	+	+	+	+	-	-	-
	4	+(2)	+	-	+	-	+	-	-
	5	-	-	+	+	-	+	-	-
	6	-	-	+	+	+	-	-	-
	9	+ or -	+	+	+	-	+	-	-
	Rough	+ or -(3)	+ or -(3)	+ or -(3)	+ or -(3)	-	-	+	+

(1) dye concentration, 20µg/ml in Blood Agar Base medium with 5% of serum (1:50,000)
(2) usually positive on primary isolation
(3) + or -, according to the original smooth type

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, 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 is considered to have an advantage over Southern blotting, since it is easier to perform and is less time-consuming when applied to large numbers of samples.

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, Cloeckaert *et al.,*(1995) using PCR-RFLP and a greater number of restriction enzymes 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 hybridisation, 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 contained in *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 contain 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). See Table 4 for PCR-RFLP patterns.

Table 4: Differential characteristics of *Brucella* species involved in sheep and goats brucellosis

Species	PCR - RFLP patterns (1) of genes (restriction endonuclease)				
	Omp25	Omp2b		Omp31	dnaK
	(EcoRV)	(AluI)	(PvuII)	(Sau3a)	(EcoRV)
B. melitensis	NC(2)	P1	NC	P1	P1
B. ovis	P1(3)	P2	P1	NC	P2
B. abortus	P2	P1	NC	NA(4)	P2

(1) For each gene, the different patterns (P1 or P2) are defined by the number and/or size of restriction fragments
(2) NC: Not cleaved
(3) *B. ovis* omp25 pattern is related to a short deletion of 36 bp in the gene
(4) NA: No amplification. *B. abortus* lacks the omp31 gene

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 acid probes, primers...) 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 completely identified. In the near future, efforts should be concentrated on the harmonisation of these

tools to propose the most suitable method for the molecular identification and typing of *Brucella.*

5.8. Conclusions

Brucellae are coccobacilli or short rods, non-motile, Gram-negative, moderate acid-fast and aerobic of which the growth characteristics are well defined. *Brucella melitensis* forms smooth (S) colonies but may dissociate in culture to rough (R) and intermediate (M) culture forms. Classical methods to identify *Brucella* include serotyping, phage typing and oxidative metabolic tests. *Brucellae* are sensitive to a broad range of antibiotics, and differences in sensitivity may be employed to help differentiate vaccine from field strains. The vaccine strain Rev.1 is sensitive to benzyl penicillin and resistant to streptomycin. All species and biovars of *Brucella* show more than 90% DNA homology. Polymorphism in some genes as identified by DNA technologies allows for differentiation.

6. THE DISEASE

6.1. Pathogenesis and immune response

Pathogenically, *B. melitensis* infection in sheep and goats is similar to *B. abortus* infection in cattle. Nevertheless, differences are significant, and each species of *Brucella* causes a different disease (OIE Manual, 1996). *Brucella* are facultative intracellular parasites of the reticuloendothelial system. The virulence of *Brucella* varies considerably according to species, strain and the size of infecting inoculum. Host susceptibility is also variable and is associated with the reproductive status. Thus, in the field, all intermediate stages between typical acute infection and complete resistance may be observed. In addition, vaccinal immunity may modify the parasite-host relationship.

6.1.1. Phases of infection

The infection in females follows a course very similar to *B. abortus* infection in cattle. The major route of infection appears to be through the mucous membranes of the oropharynx and upper respiratory tract or the conjunctiva. Other potential routes of infection are through the mucous membranes of the male or female genital tract. After gaining entrance to the body, the organisms encounter the cellular defences of the host, but generally succeed in arriving via the lymph channels at the nearest lymph node. The fate of invading bacteria is mainly determined by the cellular defences of the host, chiefly macrophages and T lymphocytes, though specific antibody undoubtedly plays a part. The outcome depends on the ruminant species infected, age, immune status of the host, pregnancy status, and the virulence and number of the invading *Brucella*. When the bacteria prevail over the body defences, a bacteraemia is generally established. This bacteraemia is detectable after 10 to 20 days and persists from 30 days to more than 2 months.

If the animal is pregnant, bacteraemia often leads to the invasion of the uterus. At the same time, infection becomes established in various lymph nodes and organs, often in the udder and sometimes in the spleen.

During this first stage of infection, the major clinical sign is abortion but other signs due to a localisation of *Brucella* may be observed (*ie*, orchitis, epididymitis, hygroma, arthritis, metritis, subclinical mastitis, etc.). However, numerous animals develop self-limiting infections or they become asymptomatic latent carriers and potential excretors. Abortion generally does not occur if the female becomes infected at the end of pregnancy.

The second stage is characterised by either elimination of *Brucella* or, more frequently, by a persistent infection of mammary glands and supramammary and genital lymph nodes (Fensterbank, 1987b) with constant or intermittent shedding of the organisms in the milk and genital secretions.

Animals generally abort once, during the mid third of gestation, but reinvasion of the uterus occurs in subsequent pregnancies with shedding in

fluids and membranes. The pregnancy can also continue to full-term. The proportion of newly infected females that abort varies with the circumstances. The percentage of infected females lambing/kidding in a flock may reach 40%. Females that are born into an infected environment and subsequently infected, generally abort less than others. This explains the high level of abortions in newly infected flocks and their relatively low frequency in flocks where infection is enzootic.

The udder is a very important predilection site for *B. melitensis*. Infection in lactating non-pregnant goats is likely to lead to colonisation of the udder with excretion of *B. melitensis* in the milk (Renoux *et al.,* 1953). In goats, about two thirds of acute infections acquired naturally during pregnancy lead to infection of the udder and excretion of the bacteria in the milk during the subsequent lactation (Alton, 1985). In some goats excretion may cease during this lactation, but in many it persists and often continues during the next (Alton, 1962). Greatly reduced milk yield follows abortion, and infection of the udder following a normal birth also leads to a considerable reduction in yield. In spite of this, clinical signs of mastitis are seldom detectable in naturally infected goats (Alton, 1990).

Sheep that abort often excrete the bacteria in the milk, but generally for not more than two months (Alton, 1990). However, exceptionally, excretion may continue for 140 days (Itabashi *et al.,* 1938) and even 180 days (Biggi, 1956).

6.1.2. Immune response

Infection with *Brucella* usually results in the induction of both humoral and cell-mediated immune responses, but the magnitude and duration of these responses is affected by various factors including the virulence of the infecting strain, the size of infecting inoculum, pregnancy, sexual and immune status of the host *(*Joint FAO/WHO Expert Committee on Brucellosis, 1986).

6.1.2.1. Humoral immunity

Following infection by natural exposure, a serological response can be expected within 2 to 4 weeks, but the response is variable and may be absent altogether. Invasion of the pregnant uterus can be expected to produce a large and persistent rise of antibodies, but this may be delayed until after abortion or parturition at the normal time. Invasion of the lactating udder causes a lesser serological response, and localisation confined to a small number of lymph nodes may fail to stimulate any response at all, or only a minimal one.

The pattern of the serological response in terms of immunoglobulin production has not been extensively studied in sheep and goats, but available information suggests close similarity to that in cattle, *ie*, production of IgM followed within a week or two by a predominance of IgG, with both isotypes falling to a low level in the more chronic stage of infection but with IgG predominating.

The serological response is transient and sometimes missing in young sexually immature animals.

As mentioned thereafter, the *B. melitensis* Rev.1 vaccine strain when applied under standard conditions (*ie* full dose via the subcutaneous route in young replacement animals) may induce a long lasting serological response to the agglutination test, that seriously interferes with serological screening for infected animals (Alton and Elberg, 1967; Elberg, 1981, 1996; Alton, 1990; MacMillan, 1990). As no differences have been found between the diagnostic antigens from field strains of *B. melitensis* and those from the Rev.1 vaccine, serological tests capable of distinguishing antibodies arising from infection and vaccination, respectively, have not been developed.

6.1.2.2. Cell Mediated Immunity (CMI)

As mentioned above, after gaining entrance into the body, the organisms encounter the cellular defences of the host and the fate of invading bacteria is mainly determined by the cellular defences of the host, in particular macrophages and T lymphocytes. The most widely used correlates of CMI are lymphocyte stimulation, macrophage inhibition, delayed-type hypersensitivity and γ–interferon induction. Like the humoral response and depending on the method used to measure the CMI, the response can be expected as rapidly as a few weeks, but the response is variable and may not be detected.

6.1.3. Conclusions

Although infection by *B. melitensis* in sheep and goats resembles infection by *B. abortus* in cattle, differences exist. *Brucella*e are facultative intracellular parasites of the reticulo-endothelial system. Virulence varies between species and strains. Infection occurs mainly through the mucous membranes of the oropharynx, upper respiratory tract, conjunctiva and genital tract. In pregnant animals the uterus is invaded resulting in abortions. The udder is an important predilection site for *B. melitensis*. Elimination of the bacteria is possible, but a long lasting persistent infection is the normal course of the disease, in particular in goats. Humoral as well as cell mediated immune mechanisms are activated in the infected animal. No differences have been found between the diagnostic antigens from field strains of *B. melitensis* and those of the Rev.1 vaccine strain. Serological tests differentiating between antibodies directed against Rev.1 and field strains have not been developed.

6.2. Clinical picture and lesions

6.2.1. Acute brucellosis

Although a variety of clinical signs has been described in artificially infected animals, the main clinical manifestations of brucellosis in sheep and goats are, as in all female ruminants, reproductive failure, *i.e.* abortion and birth of weak offspring. Abortion generally occurs during the last 2 months of pregnancy and is followed in some cases by retention of foetal membranes. In the male, localisation in the testis, epididymis and accessory sex organs is common, and bacteria may be shed in the semen. This may result in acute orchitis and epididymitis and later in infertility. Arthritis is also observed occasionally in both sexes. There is no evidence that the clinical features of *B. melitensis* infection in sheep and goats vary according to the biovar involved (Fensterbank, 1987b).

6.2.2. Chronic brucellosis

Animals generally abort once, although reinvasion of the uterus occurs in subsequent pregnancies and *Brucella* organisms are shed with the membranes and fluids. Non-pregnant animals exposed to small numbers of organisms may develop self-limiting, immunising infections or they may become latent carriers. Persistent infection of the mammary glands and supramammary lymph nodes is common in goats with constant or intermittent shedding of the organisms in the milk in succeeding lactations, while the self-limiting nature of the disease in sheep, which is seldom accompanied by prolonged excretion of the bacteria, has been observed (Alton, 1990, Durán-Ferrer, 1998). The inflammatory changes in the infected mammary gland reduce milk production by an estimated minimum of 10%. Orchitis and epididymitis generally lead to a chronic infection.

6.2.3. Macroscopic and microscopic lesions

Brucella-infected animals generally develop granulomatous inflammatory lesions which frequently are found in lymphoid tissues and organs such as reproductive organs, udder and supramammary lymph nodes and sometimes joints and synovial membranes. The lesions when present are not pathognomonic. The following could be observed: necrotising placentitis, palpable testicular alterations, necrotising orchitis and epididymitis with subsequent granuloma, necrotising seminal vesiculitis and prostatitis. Acute mastitis with palpable nodules and the production of clotted and watery milk may occur.

Some aborted foetuses may have an excess of blood-stained fluids in the body cavities, with enlarged spleen and liver. Others appear normal. Infected foetal membranes show changes affecting part or all of the membrane. The necrotic cotyledons lose their blood-red appearance becoming thickened and dull-grey in colour.

6.2.4. Conclusions

The predominant symptom of an acute *B. melitensis* infection is reproductive failure with abortion and birth of weak offspring. Abortion occurs during the last two months of gestation. In males the reproductive organs are affected and bacteria may be shed with semen. Persistent infection of the udder is accompanied by intermittent discharge of the agent in milk. Inflammation of the mammary gland reduces milk production. Aborted foetuses may show increased amounts of bloody fluids in their body cavities and enlarged spleen and liver. Foetal membranes may be oedematous or necrotic.

7. EPIDEMIOLOGY

7.1. Geographical distribution

B. melitensis infection in sheep appears to occur endemically in the Mediterranean region, especially along its northern and eastern shores, stretching through Central Asia as far south as the Arabian peninsula and as far east as Mongolia. Parts of Latin America are also seriously affected, especially Mexico, Peru and northern Argentina. The disease also occurs in Africa and India. However, North America (except Mexico) is believed to be free, as are Northern Europe (except for sporadic incursions from the south), Southeast Asia, Australia and New Zealand (FAO/OIE/WHO, 1997).

Of the three different biovars of *B. melitensis,* biovar 3 predominates almost exclusively in Mediterranean countries and Middle East, while biovar 1 seems to predominate in Latin America. The biovars 1 and 2 have also been reported in some southern European countries. However, the precise recognition of biovar 3, especially its differentiation from biovar 2 appears sometimes equivocal.

In the European Union the following Member States and regions have been recognised as being free from *B. melitensis*: Belgium, Denmark, Finland, Germany, Ireland, Luxembourg, the Netherlands, Sweden, the United Kingdom, 17 Départements of France and 2 Provinces of Spain. The disease occurs in several EU countries and the annual prevalence in those countries is given in Table 5.

Table 5: Disease prevalence in EU countries in 1998 and 1999

MEMBER STATE	HERDS			ANIMALS		
	% infected*		Difference	% infected*		Difference
	1998	1999		1998	1999	
Portugal	7.74	5.61	- 2.13	2.07	1.70	- 0.37
France	0.83	0.36	- 0.47	0.050	0.023	- 0.027
Greece**	3.35	2.34	- 1.01	0.87	0.50	- 0.37
Italy	3.21	4.71	+ 1.50	1.76	3.83	+ 2.07
Spain	17.59	18.99	+ 1.40	1.82	1.54	- 0.28

* Calculated from investigated holdings/ animals

** In Greece in 1998 vaccination in the mainland of the country commenced. The data given in this table therefore only refer to the eradication campaign on the islands (test-and-slaughter policy)

NA Not available

7.2. Modes of transmission

7.2.1. Routes of excretion and contagious material

Generally, transmission occurs in the same way in sheep and goats as in cattle, materials excreted from the female genital tract forming the main supply of organisms for transmission to other animals and man. Therefore, in most circumstances, the primary route of dissemination of *Brucella* is the placenta, foetal fluids and vaginal discharges expelled by infected ewes after abortion or full-term parturition. Very large numbers of organisms are shed at the time of parturition or abortion. In goats, excretion of the organisms from the vagina is prolonged and copious (2 to 3 months generally). In sheep excretion is generally less prolonged, usually ceasing within 3 weeks after abortion or a 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 sometimes from arthritic lesions (Alton *et al.,* 1988).

The persistent infection of the mammary glands and supramammary lymph nodes leads to a constant or intermittent shedding of the organisms in the milk in succeeding lactations. It provides an important source of infection for man and young animals.

7.2.2. Modes of infection

The modes of infection are direct or indirect. Animals become infected directly by infected aerosols or by uptake of infected material. Another mode of infection is grazing pastures where infected animals mix with brucellosis-free animals or get in touch with contaminated premises, manure, materials etc.

In the male, localisation in the reproductive organs generally results in the shedding of *Brucella* in the semen. However, when used for natural mating, the risk seems low that infected males transmit the disease to susceptible females . It should also be noted that experimental work with cattle has demonstrated that embryo transfer is unlikely to be a method of transmission, while being a useful method of preserving valuable genetic lines. Provided that recommended embryo washing and other hygienic measures are taken, embryos can be moved from infected donors to healthy recipients with minimal risk.

Dogs have been shown to be mechanical and biological vectors of brucellosis *(*Joint FAO/WHO Expert Committee on Brucellosis, 1986). Spreading via waterways is rare, and can be effective over short distances only. Persistent infection of mammary glands is associated with constant or intermittent shedding of the organisms in the milk in succeeding lactations (Philippon *et al.,*1971). The number of *Brucella* excreted in milk is relatively low but is sufficient to allow transmission to lambs and kids, and indirectly through the milker's hands.

7.2.3. Vertical transmission

Similar to *B. abortus* infection in cattle, *B. melitensis* can be transmitted from the dams to lambs or kids. A small proportion of lambs or kids can be infected *in utero*, but the majority of infections are probably acquired by consumption of colostrum or milk. These lambs or kids may have infections in the lymph nodes draining the gastro-intestinal tract and may shed *Brucella* organisms in the faeces. 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). In that case, lambs and kids remain fully susceptible when they reach sexual maturity. Despite the low frequency of transmission, the existence of latent infections greatly increases the difficulty of eradicating this disease, as *B. melitensis* persists without having a detectable immune response because of immunotolerance. The exact mechanism of the development of latent *B. melitensis* infections remains unknown (Grilló *et al.,* 1997).

In goats, the infection may vary in duration from very short periods of slight infection which is rapidly eliminated (especially in vaccinated animals) to persistence for years, where excretion of the organism in the milk may continue for two or more lactations. The situation in sheep is very different, though this varies with the susceptibility of the breed (Alton, 1990). After recovery from infection with *B. melitensis*, sheep are very resistant to re-infection (Alton, 1990). Werschilova and Striedter (1938) demonstrated a strong resistance to reinfection, even during pregnancy, up to 8 to 9 months, after which it began to decline, although some resistance was still demonstrated after 2 years. Durán-Ferrer (1998) reported a long-lasting immunity after an experimental infection.

7.2.4. Survival of B. melitensis *in the environment*

The ability of *Brucella* to persist outside mammalian hosts is relatively high compared with most other non-sporing pathogenic bacteria, under suitable conditions. Numerous studies have assessed the persistence of *Brucella* under various environmental conditions (Table 6). Thus, when pH, temperature and light conditions are favourable, *i.e.* pH>4, high humidity, low temperature and absence of direct sunlight, *Brucella* may retain infectivity for several months in water, aborted foetuses and foetal membranes, faeces and liquid manure, wool, hay, on buildings, equipment and clothes. *Brucellae* are able to withstand drying particularly in the presence of extraneous organic material and will remain viable in dust and soil. Survival is prolonged at low temperatures, especially below 0°C (Alton, G.G., 1985; Joint FAO/WHO Committee, 1986; Nicoletti, P.,1980). Contaminated equipment can be sterilised by autoclaving (121°C). Chemical treatment is recommended to destroy *Brucella* in contaminated premises. Xylene (1ml/litre) and calcium cyanamide (20 kg/m^3) have been found to be effective in liquid manure after 2–4 weeks. A 1 hour treatment with 2.5% sodium hypochlorite, 2-3% caustic soda, 20% freshly slaked lime suspension, or 2% formaldehyde solution will suffice to destroy *Brucellae* on contaminated surfaces.

The survival of *Brucella* in milk and dairy products is related to a variety of factors including the type and age of product, humidity level, temperature, changes in pH, moisture content, biological action of other bacteria present and conditions of storage. The results of some studies *(*Carrère *et al.,*1960; Davies and Casey*,* 1973; Nicoletti, 1989; Plommet *et al.,* 1988) are presented in Table 7.At low concentration in liquid media, *Brucellae* are fairly heat-sensitive. Thus, dilute suspensions in milk are readily inactivated by pasteurisation (high-temperature short-time or flash methods) or by prolonged boiling (10 min) (Davies and Casey, 1973).

Brucella do not persist for a long time in ripened fermented cheese. The optimal fermentation time to ensure safety is not known, but is estimated at 3 months *(*Nicoletti, 1989*).* However, in normally acidified soft cheese, the strictly lactic and short-time fermentation and drying increase the survival time of *Brucella* (Table 7). Previous pasteurisation of milk or cream is the only means to ensure safety of these products.

Brucella are fairly sensitive to ionising radiation and are readily killed by normal sterilising doses of gamma-rays under conditions which ensure complete exposure, especially in colostrum (Garin-Bastuji *et al.,* 1990a).

In contrast to dairy products, the survival time of *Brucella* in meat seems extremely short, except in frozen carcasses where the organism can survive for years. The number of organisms per gram of muscle is small and rapidly decreases with the pH drop of the meat. Direct contamination of abattoir workers is prevented by a proper and hygienic removal and disposal of mammary glands, reproductive organs and lymph nodes which are the most heavily contaminated. These precautions also prevent the contamination of the carcass by utero-vaginal secretions.

Most commonly available disinfectants readily kill *Brucella* in aqueous suspensions at normally recommended concentrations (phenol 10g/l, formaldehyde, xylene 1ml/l), except in the presence of organic matter or at low temperature, which drastically reduces the efficacy. Where possible, decontamination should be carried out by heat treatment, especially on surfaces. Diluted hypochlorite solutions, ethanol, isopropanol or iodophores and better, substituted phenols are effective for decontamination of the exposed skin (Joint FAO/WHO Expert Committee on Brucellosis, 1986). In contrast, the alkyl quaternary ammonium compounds are not recommended.

Table 6: Studies on *Brucella* survival time in the environment (Alton, 1985; Joint FAO/WHO Committee, 1986; Nicoletti, 1980)

Environment	Conditions	Survival time
Direct sunlight	< 31°C	4 h 30
Water	-4°C	4 mo
Water (laboratory)	20°C	2.5 mo
Water (lake)	37°C pH = 7.2	< 24 h
	8°C pH = 6.5	> 2 months
Soil	Dried in laboratory	< 4 days
	Dried at 18°C	69-72 days
	Wet	< 7 days
	Humid atmosphere	> 2 months
	Autumn (90% humidity)	48-73 days
	February (rapid drying)	72 days
Urine	37°C, pH = 8.5	16 hours
	08°C, pH = 6.5	6 days
Raw milk	25-37°C	24 hours
	8°C	48 hours
	-40°C	2.5 years
Whey	17-24°C	< 5 days
	5°C	> 6 days
Manure/dung	Summer	24 hours
	25°C	1 months
	Winter	2 months
	8°C	1 year
	-3°C	3 months
Liquid manure	Summer	3 months
	Winter	6 months
	In tank	1.5 months
	In tank (12°C)	> 8 months
Wool	In Warehouse	4 months
Hay		Several d to mo
Street dust		3-44 days
Wooden walls or floors of pens		4 months
Pasture	Sunlight	< 5 days
	Shade	> 6 days

Table 7: Studies on *Brucella* survival time in dairy products (Carrère et *al.,* 1960; Davies and Casey.,1973; Nicoletti, 1989; Plommet *et al.,*1988)

Product	Species of *Brucella*	Temperature (°C)	pH	Survival time
Milk	*B. abortus*	71.7	-	5-15 seconds
	B. abortus	38	4.00	< 9 hours
	B. abortus	25-37	-	24 hours
	B. abortus	0	-	18 months
Cream	*B. abortus*	4	-	6 weeks
	B. melitensis	4	-	4 weeks
Ice cream	*B. abortus*	0	-	30 days
Butter	*B. abortus*	8	-	142 days
Cheese:				
Various	*B. abortus*	-	-	6-57 days
Various	*B. melitensis*	-	-	15-100 days
Feta	*B. melitensis*	-	-	4-16 days
Pecorino	*B. melitensis*	-	-	< 90 days
Roquefort	*B. abortus* & *B. melitensis*	-	-	20-60 days
Camembert	*B. abortus*	-	-	< 21 days
Erythrean	*B. melitensis*	-	-	44 days
Cheddar	*B. abortus*	-	-	6 months
White	*B. melitensis*	-	-	1-8 weeks
Whey	*B. abortus*	17-24	4.3-5.9	< 4 days
	B. abortus	5	5.4-5.9	> 6 days

7.3. Susceptibility

7.3.1. Age

B. melitensis infection causes disease only in adult (sexually mature) females and males. Young animals may be infected but do not show any clinical sign and generally show only a weak and transient serological response. However, susceptibility increases after sexual maturity and especially with pregnancy.

7.3.2. Species and breed

The goat was originally considered the principal host of *B. melitensis*, notably in Latin America, where sheep are not significantly infected even when kept in close contact with goats. In many other areas the disease is more important in sheep. There are several reasons for this difference. First, whereas most breeds of goat are fully susceptible to infection, a great variation in the susceptibility of different breeds of sheep has been reported. Thus, sheep milking breeds appear more susceptible than those kept for meat production (Corbel & Brinley-Morgan, 1984). Maltese and South American sheep breeds appear very resistant, whereas the fat-tailed sheep of Southwest Asia and Mediterranean breeds are very susceptible and form a reservoir of infection that gives rise to widespread infections of man. Therefore in most countries bordering the Mediterranean Sea and in Southwest Asia, the brucellosis problem largely centres on sheep, while in Latin America goats are chiefly involved. Second, in the Mediterranean sheep are the predominant species, being often kept in large flocks, in conditions that favour the spread of infection. Species behaviour is also considered as a favouring factor. Ewes generally gather together at lambing or at night, while goats do not.

Excretion from the vagina in goats is more copious and prolonged than in cows and lasts for at least 2-3 months. In this animal, about two thirds of acute infections acquired naturally during pregnancy lead to infection of the udder and excretion of the organisms in the milk during the next lactation. Excretion may cease during a lactation. Infection in goats results in a greater reduction in milk production than is the case in cattle (Alton, 1985).

7.3.3. Individual factors

While it is obvious that individual factors influence the outcome of *Brucella* infection in small ruminants (self-limited infections, acute infection, latent persistence, etc.) no studies have demonstrated the exact nature and role of individual factors compared with other favouring or unfavourable factors.

7.3.4. Favouring factors (e.g.: husbandry, climate, season, lambing)

The system of husbandry as well as the environmental conditions greatly influence the spread of infection. Thus lambing/kidding in dark, crowded enclosures is more favourable to spread than lambing/kidding in the open air in a dry environment. The spread of infection between flocks generally follows the movement or gathering of infected animals. The main risk for introducing the disease into a previously non-infected area is by purchase of infected animals. In several countries there is a strong correlation between the prevalence of brucellosis in small ruminants and the practice of transhumance. Intermingling of flocks may occur under nomadic or seminomadic conditions of husbandry and also in static village flocks where animals are taken daily for grazing on common pastures.

7.3.5. Brucella melitensis *in other species*

Dogs

Dogs working with infected flocks frequently become infected but it has been reported that they eliminate the infection relatively quickly. Nevertheless, in certain countries, like France and Germany, it is required that when sheep or goats flocks are depopulated, shepherd dogs also must be eliminated, or at least treated with antibiotics and castrated.

Dogs, cats and wild carnivores, such as foxes and wolves, may be important as mechanical disseminators of infection by carrying away infected material such as foetuses or foetal membranes.

Cattle

In regions where *B. melitensis* is prevalent in sheep and goats, cattle may become infected from them. It has not been established whether *B. melitensis* can maintain itself indefinitely in a cattle population in the absence of infected sheep and goats. *B. melitensis* has been reported to induce either more or less abortion in cattle than *B. abortus*. Colonisation of the udder is frequent and excretion of *Brucella* in the milk may be prolonged for months or years and has frequently led to epidemics of brucellosis in people working with the cattle or drinking the milk. The serological response is similar to that observed in *B. abortus* infection. Eradication by conventional methods of test and slaughter is said to be relatively easy. However, there is little information with regard to the efficacy and innocuousness of Rev.1 vaccine in cattle.

Camels

There is serological and bacteriological evidence suggesting a high level of *Brucella melitensis* infection in camels in a number of countries, especially where these are in contact with infected small ruminants. Camel milk is a known source of infection for humans.

Pigs

Pigs are susceptible to *B. melitensis* infection from small ruminants which could easily occur in areas where pigs are bred in open-air farms (Garin-Bastuji and Hars, 2000).

Other animals

Any of the wild ruminants that come in contact directly or indirectly with infected sheep or goats seem liable to infection with *B. melitensis* and to maintain the infection in the natural environment. It has been reported in the alpine ibex in Italy (Ferroglio et al., 1998) and in chamois in the Southern French Alps (Garin-Bastuji *et al.*, 1990). In this species there is evidence of a significant clinical disease including dramatic clinical signs of orchi-epididymitis, polyarthritis, blindness and various neurological signs. No abortion has been reported and there is no evidence that these species could become a reservoir of the disease for the domestic populations.

7.4. Conclusions

Biovar 3 of *B. melitensis* is the predominant biovar in the Mediterranean countries. Transmission occurs as in *B. abortus* mainly through materials excreted by the female genital tract. The primary organ of dissemination is the placenta after abortion or full term parturition. Excretion of bacteria, especially in goats, may be prolonged. Infection may be direct through contact with contaminated material or aerosol infection, or indirectly by grazing on contaminated pastures or through other materials. Dogs may be vectors mechanically or biologically. Milk plays a minor role in transmission. Lambs and kids can become infected in utero. In goats, infection can vary from a short time to persistence for years. In sheep, the course of infection depends upon the dose of infection. After recovery sheep are resistant to reinfection. Brucellae show a high degree of resistance outside the mammalian hosts to inactivating agents. Favouring factors are pH >4, high humidity, low temperature and no sunshine. Disinfectants are effective to inactivate the agent on contaminated surfaces. Brucellae do not appear to survive in matured cheese stored for at least three months. Pasteurisation of milk ensures safety of dairy products. *B. melitensis* causes disease only in adult animals. Male and female animals are equally susceptible. Most breeds of goats are fully susceptible but the susceptibility of sheep breeds differs widely. Individual factors influence the outcome of an infection. The husbandry system, as well as environmental conditions, affects the spread of infection. Mediterranean sheep breeds are very susceptible and form a reservoir giving rise to widespread infections in man. Dogs and some wild carnivores may carry the infection to other places. Cattle, pigs and wildlife ruminants can become infected.

8. DIAGNOSTIC TESTS

8.1. Direct diagnosis: agent detection

8.1.1. Isolation of bacteria

The only unequivocal method for the diagnosis of brucellosis in small ruminants is based on the isolation of *Brucella* bacteria. (Alton *et al.,* 1988). The presumptive bacteriological diagnosis of *B. melitensis* can be made by means of the microscopic examination of smears from vaginal swabs, placentas or aborted foetuses stained with the Stamp modification of the Ziehl-Neelsen method. However, morphologically related micro-organisms such as *B. ovis*, *Chlamydia psittaci* or *Coxiella burnetii* can mislead the diagnosis. Accordingly, the isolation of *B. melitensis* on appropriate culture media is recommended for an accurate diagnosis. Vaginal swabs and milk samples are the best samples to isolate *B. melitensis* from sheep and goats. The spleen and lymph nodes (iliac, mammary and prefemoral) are the most reliable 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 nonselective media cannot be recommended because of the overgrowing contaminants usually present in field samples, and selective media are needed for isolation purposes. The Farrell's 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, may have inhibitory effects on 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's modified medium (Marín *et al.,* 1996a). The sensitivity of bacteriological diagnosis is significantly increased by the simultaneous use of both the Farrell's and the modified Thayer-Martin's 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.

8.1.2. Other methods for agent detection

While culturing is a specific method, its sensitivity depends on the viability and numbers of *Brucella* within the sample, the nature of sample (foetal organs, foetal 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. Thus, in the case of tissues or fluids contaminated with non viable or a low number of *Brucella*, PCR could be a potentially useful method for the diagnosis of brucellosis. Several authors reported a good sensitivity of PCR for detecting of *Brucella* DNA on pure cultures (Fekete *et al.,*1990a, 1990b; 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). The possibility of PCR techniques to detect the DNA of dead bacteria, or in paucibacillary samples and even in samples highly contaminated with other micro-organisms, could potentially increase the rate of detecting animals infected with *Brucella.* 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) and up to now, no technique has been demonstrated to be sensitive enough to replace classical bacteriology on all kinds of biological samples.

8.1.3. Conclusions

A preliminary bacteriological diagnosis can be made on smears from vaginal swabs, milk, placentas or aborted foetuses stained with Stamp's method. Confirmation on appropriate culture and selective media is recommended. Spleen and lymph nodes are most reliable from necropsy material. PCR is potentially a useful method in samples containing a low number of Brucella.

8.2. Indirect diagnosis: antigens of *Brucella* and antibody detection

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 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). They are currently the official tests used in member states of the European Union (Council Directive 91/68/EEC). The antigen 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 that the sensitivity of the classical RB antigen prepared with *B. abortus* biovar 1 (A-dominant) was adequate for diagnosing ovine populations infected with the M-dominant *B. melitensis* biovar 1 (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. Most serological tests, particularly those using whole-cell suspensions as antigen (such as RB, CF), as well as ELISA , 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-mannopyranoside 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) have shown 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 tetrasaccharide 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 (*ie B. abortus* biovar 1) at detecting M-dominant *B. melitensis* biovar 1 infections and *vice-versa* (MacMillan, 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 (Alonso-Urmeneta *et al.,* 1998). However, the native hapten and the S-LPS hydrolytic polysaccharides containing the O-chain and core sugars from *B. abortus* biovar 1 failed 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 with *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 opportunistic human pathogen, has been reported recently (Velasco *et al.,* 1997). The *Brucella* cytoplasmic antigens, 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 uncharacterised 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, 1996b). 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 showed improved 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, 1994b, 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 as potential antigens for the diagnosis of brucellosis in sheep by immunoblotting or ELISA, the antibody response to them was very low and heterogeneous in *B. melitensis* infected sheep (Zygmunt *et al.,* 1994a, 1994b).

Further research is needed on the identification, isolation, characterisation 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.

8.2.1. Conclusions

Antigens for the rose Bengal test and the CF test are prepared from cultures of *B. abortus*. Antigens reactive in serology are included in the outer membrane of the bacteria. Smooth lipopolysaccharide (S-LPS) is the immuno-dominant antigen. Most tests detect this type of antigen. Crude LPS extracts from either B. abortus or *B. melitensis* are equally sensitive in an indirect ELISA to diagnose *B. melitensis* infections in sheep. Some cytosoluble proteins of *B. melitensis* have been reported able to differentiate Rev.1-vaccinated from wildtype infected sheep. In precipitin tests, antigen

prepared from *B. melitensis* is superior. Cytoplasmic antigens are components of brucellin which is used in allergic tests.

8.3. Serological tests

Considering that infections by *B. abortus* and *B. melitensis* cause two different diseases in any of the susceptible host species, it is striking that no specific serological tests for *B. melitensis* infection of sheep have been developed. Instead, 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 test are the most widely used tests for the serological diagnosis of brucellosis in sheep and goats (Farina, 1985; Alton, 1990; MacMillan, 1990). There is a considerable body of knowledge available on the diagnostic value of tests such as iELISA, cELISA, FPA, Coombs test or immunocapture test for ruminants in general. However, such knowledge does not enable to make a clear choice regarding the selection of a test for use in the serodiagnosis of ovine and caprine brucellosis (OIE Manual, 2000).

8.3.1. Rose Bengal Test

The RB test was developed more than 20 years ago for the diagnosis of bovine brucellosis. 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, 1994b), 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 standardisation of the antigen. 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 give a negative reaction at a dilution of 1:55 (18.2 IU/ml) of the same serum (Council Directive 64/432/EEC, 1964). These standardisation conditions, which seem to be suitable for the diagnosis of *B. abortus* infection in cattle (MacMillan, 1990), limit the sensitivity of the test resulting in reduced performance for the diagnosis of *B. melitensis* infection in sheep (Blasco *et al.,* 1994a, 1994b). This accounts for the relatively low sensitivity of some commercial RB antigens when 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 test (Blasco *et al.,* 1994a). These phenomena have raised serious questions over the efficacy of using the RB as an individual test in small ruminants. However, if the antigen is standardised differently to give a higher analytical sensitivity, the diagnostic sensitivity is much improved (MacMillan, 1997). Some workers claimed that, at least for sheep, the sensitivity of the RB test can be improved significantly when the antigens are standardised against a panel of sera from several *B. melitensis* culture positive and *Brucella*-free sheep, respectively, or when the volume tested is increased from 25µl to 75µl (Blasco *et al.,* 1994a).

8.3.2. Complement Fixation Test

The CF test 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 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 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). More recently (Nielsen *et al.,*2000), in a Pan-American and European comparative study, the results on sensitivity for the different tests were: cELISA (76.0 %), buffered plate agglutination test (77.5 %), CFT (83.1 %), iELISA (90.1 %) and fluorescence polarisation assay – FPA (91. 5%). 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 titres. 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).

8.3.3. Enzyme-immunoassay (EIA) Methods

The large majority of EIAs in use in brucellosis diagnosis are indirect ELISAs (iELISA). ELISAs are methods that involve the immobilisation of one of the active components on a solid phase, and iELISAs are those in which the antigen is bound to a solid phase, usually a polystyrene microtitre plate so that antibody, if present in a sample, binds to the immobilised antigen and may be detected by an appropriate anti-globulin-enzyme conjugate which in combination with a chromogenic substrate gives a coloured reaction indicative of the presence of antibody in the sample. It is this method that is now familiar to most diagnosticians.

Another method which is gaining prominence in the publications on brucellosis diagnosis is the competitive ELISA (cELISA). (Gorrell *et al.,* 1984 ; Rylatt *et al.,* 1985 ; Sutherland *et al.,* 1986 ; Macmillan *et al.,* 1990 ; Greiser-Wilke *et al.,* 1991 ; Nielsen *et al.,* 1991 ; Marín *et al.,* 1999; Nielsen *et al.,* 2000). In this test, *Brucella* antigen is immobilised on the plate as with the indirect ELISA. Following that, the serum under test and a monoclonal antibody directed against an epitope on the antigen are co-

incubated. This anti-*Brucella* monoclonal antibody is conjugated to an enzyme, the presence of which is detected if it binds to the antigen. This will only occur if there is no antibody in the serum sample which is bound preferentially.

The Particle Concentration Fluorescence Immunoassay (PCFIA) is a commercially produced immunofluorescence assay which has been used in the USA and elsewhere (Nicoletti and Tanya, 1993; Greenlee *et al.,* 1994; Reynolds 1987).

The technique has much in common with the competitive ELISA in that antigen is immobilised onto polystyrene, except that in this case it is initially in the form of sub-micron particles held in suspension. Serum is co-incubated with an anti-*Brucella* conjugate in the presence of the antigen coated particles, and they compete for binding. In this case, the antibody is labelled with a fluorescent probe. The unreacted reagents are separated from the polystyrene particles which are themselves concentrated by filtration through a membrane at the bottom of each well. The commercial producer claims the ability to analyse 550 samples per hour with that system.

The term ELISA covers a variety of possible reagent combinations which have a direct and significant effect on performance. A wide variety of antigen preparations has been used in the iELISA ranging from whole cells to crude and semi-purified smooth lipopolysaccharide (sLPS) preparations to polysaccharides and proteins. The antigens of sLPS are the most immunodominant and, due to the fact that they are very strongly adsorbed to polystyrene, have become the antigens most widely used for routine diagnosis. Even in relatively crude extracts or when whole cells are used, it is the sLPS antigens which are most active.

The diagnostically most relevant epitopes reside on the chain component rather than the lipid A or core polysaccharides. The O-chain structure of all species of *Brucella* has been elucidated and has been shown to be a linear homopolymer of 4,6-dideoxy-4-formamido-α-D-mannose. (Perry *et al.,* 1990; Bundle *et al.,*1987; Cherwonogrodzky *et al.*1984; Miekle *et al.*1989)

In practice, the use of an sLPS antigen derived from either *B. abortus* or *B. melitensis* is adequate for the diagnosis of either, but there is some evidence that it is preferable to use a homologous antigen. There is little evidence for any significant difference between antigens prepared from *B.abortus* or *B. melitensis*, whether LPS, O-polysaccharide or native hapten is used for the detection of brucellosis in vaccinated or non-vaccinated cattle (Nielsen *et al.*1983, Devi *et al.*1987).

Although it is recommended that purified sLPS antigen should be used in the iELISA, there is little doubt that less purified preparations such as autoclaved or sonicated cell extracts are adequate, at least for screening purposes. (Cherwonogrodzky *et al.,* 1986; Nielsen *et al.,* 1988)

There is also consensus that the purity of the antigen has little effect on the sensitivity of the assay, but as diagnostic specificity becomes increasingly important with decreasing disease prevalence, it can be expected that less

pure antigens give rise to a slightly higher number of false positive reactions. On the other hand, it is suspected that extremely pure sLPS has the tendency to form micelles in aqueous solution causing it to adsorb irregularly to polystyrene. This results in assay variation and high negative backgrounds. The hot water/phenol extraction method appears to be one of the best methods currently in use.

All four IgG isotypes are present in serum and milk although IgG1 predominates. All isotypes are theoretically detectable in the iELISA depending on the immunological specificity of the anti-globulin used, and a wide variety of conjugates have been evaluated ranging from those of broad specificity to reagents specific for a single isotype. The use of anti-γ globulin reagents introduces a bias in favour of IgM, a class of antibody often associated with non-specific reactions in other assays. This bias can be reduced by using an anti-IgG (heavy and light chain) which would still be capable of detecting IgM as a result of its light chain specificity but would favour the detection of IgG isotypes which are likely to be more specific (Nielsen *et al.*1983, 1984, 1988; Huang ,1987; Lamb *et al.,*1979; Wright and Nielsen, 1986).

The diversity and multiplicity of epitopes on sLPS make it likely that the immune response to this antigen will be very heterogeneous in terms of relative specificities and affinities. This has significance in that there will be competition for a limited number of binding sites between antibodies of different isotypes. There is undoubtedly competition both directly at a single epitope, but also as a result of the structure of the O-chain, by steric hindrance between antibodies binding to adjacent or overlapping sites. Thus a conjugate of high specificity for a single isotype may fail to detect the presence of antibody if this isotype is prevented from binding to the antigen due to competition with other isotypes. This would lead to a false negative test result.

The vast majority of ELISAs involve a common methodology utilising 96 well polystyrene plates. In the context of discussions on mass screening as part of eradication campaigns this is a progress as it is suitable for cost effective automation.

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 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 IgG_1 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, Marín and Moriyón, unpublished results). Literature on the use of competitive ELISA (c-ELISA) protocols for the diagnosis of brucellosis in sheep is 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, Marín, Moriyón and Blasco, 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; E Moreno, CM Marín, I Moriyón and JM Blasco, 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).

While the use of ELISA for the diagnosis of bovine brucellosis is well developed, more extensive field trials are required to fully validate the test for use in sheep and goats.

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

8.3.4. Gel Precipitation Test

As with the RB and CF tests, the specificity of the i-ELISA is quite low when testing sera from sheep and goats subcutaneously vaccinated with the live *B. melitensis* Rev.1 vaccine (Jiménez de Bagués *et al.*, 1992, Díaz-Aparicio *et al.*, 1994, Marín *et al.*, 1999). Under these conditions, only the Gel Diffusion (GD) or the Radial immunodiffussion (RID) tests with Native Hapten (NH) as antigen (Díaz and Moryion., 1989) are specific enough to discriminate the immune responses of, respectively, sheep (Marín *et al.*, 1999) and goats (Díaz-Aparicio *et al.*, 1994) infected with *B. melitensis* from those due to Rev.1 vaccination. As demonstrated by the contrasting results of the i-ELISA and the c-ELISA, the superior diagnostic specificity of the latter in vaccinated sheep (Marín *et al.*, 1999) can be due to the elimination by the competing anti-C monoclonal antibody of the low avidity antibodies, supposed to be dominant in the sera from vaccinated animals. This antibody avidity, rather than epitopic differences in the antigens used, is also likely to account for the high specificity of the NH precipitation tests. The higher specificity of the precipitation tests with NH may result from the higher threshold avidity required in precipitation tests as compared to that of i-ELISAs (Peterfy *et al.*, 1998). This could explain why NH fails to react with sera from vaccinated animals in the former but not in the latter assay, if low avidity antibodies are predominant at a given time after vaccination. Obviously, comparison of the results of the indirect and competitive ELISAs indicates that the sera of Rev.1 vaccinated sheep, at a given time after vaccination, contain mostly antibodies of lower avidity than those from infected sheep, and this is consistent with the hypothesis proposed.

Many serological responses occurring in healthy animals (particularly in the Rev.1 immunised ones) in endemic areas may be the consequence of mere secondary antigenic contacts and do not correspond always to established infections in the host. In fact, under these conditions, the RB, CF and i-ELISA tests have been reported to be highly unspecific to detect exclusively the bacteriologically positive animals (Blasco *et al.*, 1994b). Accordingly, an overkilling of healthy animals is currently taking place in the present eradication campaigns in EU countries, with the ensuing disagreement among veterinarians and owners. Having in mind that more additional research and field studies are needed for a proper confirmation, the NH precipitation test (Díaz and Moryion., 1989, Díaz-Aparicio *et al.*, 1994, Marín *et al.*, 1999) again appears to be the most specific serological test to detect exclusively the epidemiologically relevant animals, particularly after Rev.1 mass vaccination programmes in endemic situations. The avidity hypothesis commented above could account for the high specificity of the NH tests to detect exclusively the truely infected animals. Additional research should be conducted to validate these NH precipitation tests and determine their practical usefulness in eradication campaigns.

8.3.5. Antiglobulin (Coombs') Test

The antiglobulin test or Coombs' test (Coombs *et al.,* 1945) was developed to detect antibodies which, although they combine with cellular antigens of *Brucella,* do not give rise to agglutination. The presence of these so-called "incomplete agglutinins" can be detected by using an antibody directed against the IgG fraction of the animal species being tested (MacMillan, 1990). Early in 1955, the test was described as the most effective immunological method for the detection of brucellosis in goats (Esteban, 1959). The classical time-consuming methodology of the test in man has been considerably improved by its adaptation to a microtiter plate format (Otero *et al.,* 1982).

Farina (1985) reported that the Coombs' test could be useful to check sera from animals that give negative, suspicious or non-conclusive responses to SAT, due to the presence of incomplete antibodies in these sera. There is evidence (Alton, 1990; Unel *et al.,* 1969) suggesting that the antiglobulin test is effective in diagnosing brucellosis in sheep and also in goats, but because of the complexity of the technique its use may be restricted to special situations, e.g. for the detection of antibodies in anticomplementary sera. Its use is not recommended in bovines vaccinated with strain S19 (MacMillan, 1990) or in small ruminants immunised with Rev.1 vaccine (Farina, 1985), because of its low specificity as compared to the CFT.

8.3.6. Immunocapture Test

The immunocapture test is a one step technique, very easy to perform, able to detect antibodies of medium to high affinity against *Brucella*, and suitable for simple standardisation and automation. The test is based on a blue-coloured cellular antigen of *Brucella melitensis* (strain 16M) and on anti-total species immunoglulin coated polystyrene microtiter plates of 96 U-wells.

The test was initially evaluated for the serodiagnosis of human brucellosis and has shown high sensitivity and specificity both in the first stages of the disease and, in particular, in chronic cases as well as in relapses and reinfections (Gómez *et al*., 1999; Orduña *et al.,* 2000). Moreover, the immunocapture test and Coombs' test have a similar performance in the diagnosis of human brucellosis but the immunocapture test is more sensitive than the Coombs' test and usually shows higher titers (Orduña *et al*., 2000).

Recently, the usefulness of the immunocapture test for the recognition of *Brucella melitensis* infection in sheep has been evaluated in experimental models of vaccination and infection (Mendoza *et al.,* 2000). The test revealed an optimal sensitivity in the detection of animals with active brucellosis (abortion and excretion) and adequate specificity in Rev.1 immunised animals that were protected against challenge with the standard virulent strain 53H38 of *B. melitensis*. Moreover, the specificity of the immunocapture test was higher than RBT and CFT either in lambs or in adult animals vaccinated with Rev.1 by conjunctival route.

Field studies are being conducted to evaluate the performance characteristics (sensitivity, specificity, repeatability, and reproducibility) of the immunocapture test either in small ruminants or in cattle.

8.3.7. Fluorescence Polirisation Assay (FPA)

The principle of fluorescence polarisation was described by Perrin in 1926. It is a simple technique for measuring antigen/antibody interaction and may be performed in a laboratory setting or in the field. It is a homogeneous assay in which analytes are not separated and it is therefore very rapid (Corbel and MacMillan, 2000).

The premise for the Fluorescence Polarization Assay (FPA) is that a small molecule in solution randomly rotates at a rate inversely proportional to its size. Plane polarized light allows measurement, using an attached fluorochrome, of the rate of rotation through a given angle. Thus a small molecule rotating at a high rate will revolve through the angle rapidly, resulting in a low polarization value. If an antibody is attached to the small molecule, the increased size will cause a decrease in the rate of rotation, resulting in a higher polarization value. The FPA is a homogeneous assay, requiring no steps to remove unreacted reagents, and can therefore be performed in minutes, even outside the laboratory, and is very cost effective.

Based on this, Nielsen *et al.,* (1996) developed the FPA for the detection of antibodies to *Brucella abortus* in cattle, using as antigen a small molecular weight fragment of O-polysaccharide prepared from *B. abortus* lipopolysaccharide (average 22 kDa) conjugated with fluorescein isothiocyanate. This antigen is added to diluted serum or whole blood and a measure of the antibody content is obtained in about 2 minutes using a fluorescence polarisation analyser.

The sensitivity and specificity values of the FPA for bovine brucellosis are almost identical to those of the cELISA (Corbel and MacMillan, 2000). The diagnostic sensitivity has been determined to be over 99 %, while the

diagnostic specificity approaches 100%. The diagnostic specificity for cattle recently vaccinated with *B. abortus* S 19 is over 99 % (Nielsen *et al.,* 1996). The test has been evaluated with positive outcomes under field conditions in different epidemiological situations (Nielsen *et al.,* 1996, 1998; Dajer *et al.,* 1999). The technique has also been reported to be a valuable asset to the diagnosis of porcine brucellosis (Nielsen *et al.,* 1999); the sensitivity (93.5 %) was estimated much higher than for cELISA (90.8 %) and also the specificity (97.2 % versus 96.6 %).

Little information is available on the usefulness of the FPA for the serodiagnosis of ovine and caprine brucellosis. In a preliminary study comparing different serological tests for ovine brucellosis (Nielsen *et al.,* 2000), the sensitivity of the FPA (91.5 %) was estimated as higher than other tests (BPAT, 77.5 %; CFT, 83.1 %; iELISA 90.1 %, and cELISA 76.1 %). The specificity of FPA (99%) was similar to that of the other tests. Residual vaccinal antibodies would not cause reactivity in most cases in the cELISA and in the FPA. Based on these data, the FPA appears to be as efficient as other commonly used serological tests and may prove to be a useful tool for the presumptive diagnosis of *B. melitensis* infection in small ruminants (Nielsen *et al.,* 2000). It is important to note that full validation of the technique is needed under European conditions before recommendations on its use can be made.

8.3.8. Cross-reactions in serological tests

Where *B. ovis* and *B. melitensis* coexist in sheep populations, the question of cross-reactions is of considerable importance. The immunochemistry of smooth and rough *Brucella* antigens has been discussed in 8.2. but, unfortunately, there is little information available how these factors affect the routine diagnosis of *B. melitensis* infection. The smooth *Brucella* antigens used in classical tests depend on S-LPS, and therefore, they should not react with antibody against the rough *B. ovis*. However in iELISA tests preliminary reports suggest that extensive cross-reaction may occur.

The allergic test and some gel diffusion tests are mediated by protein antigens which are common to rough and smooth *Brucella* whatever the species, and therefore these tests should be positive in sheep infected with *B. ovis* and *B. melitensis*, respectively. These problems should not arise in testing goats since they are not affected by *B. ovis* in nature.

In both sheep and goats, infection with *Yersinia enterocolitica* O:9 is likely to cause cross-reactions in serological tests with smooth *Brucella* antigens in the same way as in cattle. Such infection has been identified in several countries, particularly in France (Garin-Bastuji, unpublished results). So far, little has been reported concerning cross-reactions due to other bacterial infections. In the same way as in cattle, an allergic skin test using S-LPS-free brucellin, being specific for the genus, could be useful in distinguishing reactions caused by bacteria of the genus *Brucella* from those due to other bacteria.

8.3.9. Conclusions

No specific serological test for *B. melitensis* infections of small ruminants has been developed, and it is widely assumed that serological tests used for B. abortus infections in cattle are adequate for the diagnosis of brucellosis in small ruminants. Rose Bengal and CF tests are most widely used. Other tests are the indirect ELISA, competition ELISA, plate agglutination test, fluorescence polarisation assay (FPA), Coombs test and immunocapture test. It is unclear which is the method of choice for the serological diagnosis of brucellosis in small ruminants. The purity of antigens has little effect on the sensitivity of the iELISA but non-purified antigens give rise to more non-specific reactions. Cross reactions between *B. abortus* and *B. melitensis* in sheep can be important since in both tests S-LPS are used as antigens. *B. ovis* infections caused by rough (R) Brucella are not detected. Infection of sheep and goats with *Yersinia enterocolitica* O:9 is likely to cause cross reactions in serological tests with smooth Brucella antigens.

8.4. Validation and Standards

The criteria to be used for the validation of diagnostic tests are set by the OIE (Jacobson, 1988). Strictly speaking, no fully validated test exists for the serodiagnosis of *B. melitensis* infection, as tests were transfered from those used for the serodiagnosis of *B. abortus* in cattle. None of the tests in long-standing use has been validated using modern criteria although their capabilities and limitations are well recognised through experience. There is a need for new tests to be fully validated to OIE standards before their official acceptance into use in the European Community. A Community Reference Laboratory, which could carry out this activity, is not currently established for brucellosis in sheep and goats. The National Reference Laboratories should form a network to ensure test harmonisation including the operation of ring tests organised by a Community Reference Laboratory.

8.4.1. International standards

Tests, especially when they are to be used as the basis for national eradication schemes or for international trade, must be standardised. Standardised tests provide a high degree of confidence and allow inter-laboratory harmonisation.

The basis of test standardisation is the existence of international and national Standard Sera. International Standard Sera are primary reference standards which act as reference materials for the calibration of test methods and reagents and as prototypes for the calibration of national and working standards. The current OIE recommendation is that at least three Standard Sera should be produced: a strong positive and a weak positive and a negative standard serum. Such positive standards should be produced from sera from animals exhibiting a typical immune response.

There are currently four International Standard Sera for brucellosis diagnostic tests. The Second International anti-*B. abortus* Serum was established many years ago and is used to standardise the classical serological tests. It is also currently used under Council Directive

64/432/EEC for the standardisation of the bovine ELISA. A Strong Positive, a Weak Positive and a Negative OIE Standard Serum have recently been adopted as Primary Reference Standards for the ELISA and their use for the standardisation of this test is described in the OIE Manual of Standards (2000). OIE Standards for *B. melitensis* diagnosis will shortly be established.

As Primary Reference Standards, these reagents are intended to be used by National Reference Laboratories to establish National Standards which are then used in each country to standardise commercial tests and antigens.

8.4.2. Conclusions

There is currently no specifically validated test for the serological diagnosis of *B. melitensis* infection in small ruminants as the tests were transferred from those used for the diagnosis of *B. abortus* in cattle. It is essential that comparison trials of classical and newly developed tests are carried out to validate tests for diagnosis in sheep and goats. International standards have been set by the OIE based on panels of internationally accepted national standard reference sera. It is essential that EU procedures be consistent with OIE recommendations and that new tests be fully validated according to OIE standards before their official acceptance into use in the European Community. A Community Reference Laboratory and a network of National Reference Laboratories is required to co-ordinate this activity and to ensure international harmonisation of tests and procedures.

8.5. Tests based on Cell Mediated Immunity

8.5.1. Brucellin test

Brucella cytoplasmic antigens, known also as brucellin (Jones *et al.,* 1973) have been used with variable success 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). Cold saline protein extracts of the rough *B. melitensis* 115 a strain devoid of the O-chain polysaccharide were used as allergen (Jones *et al*., 1973).

Most authors consider that the site and route of allergen inoculation is not important for DTH sensitivity (Alton, 1990; Fensterbank, 1985). The method considered efficient for sheep is the subcutaneous inoculation in the lower eyelid of 50 µg of allergen suspended in a volume of 0.1 ml saline, with readings taken 48 h after inoculation (Alton *et al*., 1988; Fensterbank, 1985; Jones *et al*., 1973). However, the intensity of the DTH responses peaks 72 h after inoculation (Blasco *et al.*, 1994). As mixed DTH-antibody mediated intradermal reactions can occasionally be observed (Blasco *et al.*, 1994), the antibody-mediated responses should be more apparent at shorter times and, accordingly, reading time longer than 48 h seems to be advisable for a better assessment of true DTH reactions. A relatively important anergic state can be induced by repeated skin testing and this should be taken into account if the DTH test to *Brucella* has to be used as a diagnostic test in the field. The DTH sensitivity is generally higher than that of the classical RB and CF serological tests (Ebadi and E. Zowghi, 1983; Trap and Gaumont, 1982;

Blasco *et al*., 1994). Although DTH has a higher sensitivity than RBT for diagnosing culture positive animals, and its use in some circumstances as a screening test in the field could be more practical than that of a serological test, a high proportion of culture negative animals belonging to infected flocks have been reported to be DTH positive (Blasco *et al.*, 1994). With respect to specificity, it is generally acknowledged that DTH tests show no positive reactions (100% specificity) when testing *Brucella*-free flocks (Alton, 1990; Dubray, 1985a & b; Fensterbank, 1985). However, the *B. melitensis* 115 allergen also causes DTH reactions in rams infected with *B. ovis.* Moreover, it cannot distinguish whether an animal is infected by *B. melitensis* or vaccinated with Rev.1. Accordingly, the DTH results should be carefully interpreted in countries where animals are infected by *B. ovis*. This test is not suitable in countries in which vaccination programmes with Rev.1 are being applied.

As it has been commented above, infection with *Yersinia enterocolitica* O:9 is likely to cause cross-reaction in serological tests with smooth *Brucella* antigens in both sheep and goats, as it is the case with cattle. Such infection has been identified in several countries, particularly in France (B. Garin-Bastuji, personal results). As in the case of cattle, the allergic skin test using S-LPS-free allergen could be useful to differentiate serological reactions caused by bacteria of the genus *Brucella* from those due to another bacteria.

8.5.2. Interferon-gamma-test

Tests for the *in vitro* detection of Cell Mediated Immunity (lymphocyte transformation and proliferation assays) showed a lack of acceptable efficacy in order to be applied for the large scale routine diagnosis of *Brucella* infection (Nicoletti and Winter, 1990).

However, in the last decade, the recognition of the role of some cytokines, such as interferon (IFN) gamma, in immunity against intra-cellular agents has enabled the development of an *in vitro* test with useful diagnostic applications. The test was first designed for the diagnosis of bovine tuberculosis (Rothel *et al.,* 1990, Wood *et al.,* 1990, 1991).

Brucellae are facultative intracellular bacteria that survive and replicate in both phagocytic and non-phagocytic cells (Dubray, 1985). Phagocytes play a key role in initiating T-cell responses by processing and presenting antigens. In this path, IFN-gamma is one of the most important T-cell stimulated cytokines in the course of an infection. It is a potent activator of macrophages and monocytes and up-regulates their metabolic activities to produce oxidative metabolites and other microbicidal molecules. Based on these data, the usefulness of IFN-gamma assay in the diagnosis ovine brucellosis (Garrido, 1992; Durán-Ferrer, 1998) and bovine brucellosis (Weynants *et al.,* 1995) has been explored.

The performance of the IFN-gamma test is divided in two phases: firstly, cultures of whole blood samples from donors are stimulated with specific antigen, and then IFN-gamma released is measured by immunodetection with a sandwich ELISA. In principle, such versatile design could lead to the

use of the test for different purposes by using different stimulating antigens (S-LPS, cytosolic proteins).

Studies of Weynants *et al.,* (1995) pointed to IFN-gamma with cytosolic proteins as a candidate stimulant tool for the detection of false positive reactions in serological tests, which have become a major problem in the surveillance of bovine brucellosis.

With regard to ovine brucellosis caused by *B. melitensis*, the test has been evaluated in experimental models of conjunctival vaccination (De Frutos *et al.,* 1994) and in experimental infection (Durán-Ferrer, 1998) of ewes, either with surface antigens or cytosol proteins. The experiments revealed that this test may be superior to serological tests (RBT, FC and iELISA) for the detection of acute infection in vaccinated ewes, but show similar specificity.

Nevertheless, the available information on the application of the test for the indirect diagnosis of *Brucella* infection is still scarce. Further research is needed for the accurate estimation of performance characteristic of the test (sensitivity, specificity, repeatability and reproducibility) and its usefulness under various epidemiological conditions.

8.5.3. Conclusions

The brucellin test is an allergic test which has been applied in sheep. It is described in Chapter 3.3.2 of the OIE Manual of Diagnostic Tests. A gamma-interferon test has been evaluated experimentally but requires further research.

9. VACCINES AND VACCINATION FOR *B. MELITENSIS* IN SHEEP AND GOATS

Vaccination is often the first step in the control of infectious diseases. There is agreement that vaccination of sheep and goats is the only practical and effective procedure to reduce the incidence of brucellosis in many countries. In most developing countries and in some EU countries affected by *B. melitensis*, the vaccination of sheep and goats continues to be applied in order to control the disease.

9.1. *Brucella melitensis* Rev.1 vaccine

The live attenuated *B. melitensis* Rev.1 strain is presently recognised as the best available vaccine for the prophylaxis of brucellosis in sheep and goats. Numerous independent field and controlled experiments confirm its value for this purpose (for a review see Alton and Elberg, 1967; Alton, 1990; Elberg, 1981 and 1996; Blasco, 1997). Moreover, "correctly standardized Elberg 101 strain Rev.1 vaccine should continue to be considered as the basis of brucellosis control in small ruminants where vaccination is applied, until new safer and effective versions of *B. abortus* and *B. melitensis* vaccines, based on rough strains, are tested under controlled experimental and field conditions and shown to be at least equivalent to the Rev.1 vaccine." (WHO, 1997). The ability of the vaccine (Rev.1 strain) to produce a high level of immunity against both artificial and natural challenge has been convincingly demonstrated both for sheep and goats (Alton, 1990).

It has been well established that a large proportion of vaccinated animals is protected against infection (Elberg, 1959, quoted by Garrido, 1992), and in those vaccinated animals where infection occurred, it is often transitory. Hence, the period of *Brucella* excretion from the udder or vagina is shorter, the degree of microbial contamination of the surroundings is reduced and, consequently, disease transmission within and between herds is significantly reduced (Garrido, 1992).

As with all highly-contagious diseases, the effect of vaccination increases the greater the coverage of the animal population. Erratic administration of vaccines or their use without adequate quality control is not effective. Adequate protection is only possible if the vaccine quality is good and if the vaccines are administered to at least 80 % of the animals at risk (Garrido, 1992).

The duration of immunity conferred by vaccination with Rev.1 was investigated by vaccinating Maltese goats when they were 4 to 12 months of age and challenging some at 2 ½ years (Alton, 1966) and others at 4 ½ years (Alton, 1968) after vaccination. Those challenged at 4 ½ years were as resistant as those challenged at shorter intervals after vaccination, and it was concluded that immunity could be considered lifelong. Similar results were observed in sheep in Iran challenged 2 ½ years after vaccination (Biggi, 1956; Alton, 1990).

More recent work has demonstrated the efficacy of Rev.1 vaccine in sheep either vaccinated as lambs (CJ or SC route) and challenged 9-10 months (Fensterbank *et al*., 1985) or 7.5- 15.5 months later, respectively (Verger *et al*., 1995), or vaccinated as adults (CJ route) and challenged 2 ½ years after (Durán-Ferrer, 1998). Likewise, good results of protection were obtained when young goats were vaccinated at 4 months of age (CJ or SC route) and challenged 8.5-12.5 months after (Fensterbank *et al.,* 1987).

The Rev.1 vaccine is a useful tool for the control of brucellosis in sheep and goats and to stop the infection of human beings. Its administration should be related to the epidemiological situation in order to be compatible with an eradication policy based on test-and-slaughter.

The degree of attenuation of Rev.1 strain is not enough to allow its use without any restriction. Due to residual virulence it may induce abortions and also lead to persistent immune responses , which could interfere with classical methods of serological diagnostic tests . With the general aim to minimise these adverse effects, different procedures for the administration of the vaccine (conjunctival route instead of subcutaneous route and/or reduction of the dose of vaccine) have been studied in the recent decades.

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 population (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 Rev.1 vaccination of lambs combined with the test-and-slaughter of adults. Finally, it may be possible to use a test-and-slaughter programme only (Garin-Bastuji *et al*., 1998).

9.2. Vaccination with reduced doses of Rev.1

As described in detail below, the vaccination of pregnant animals with a full Rev.1 dose administered subcutaneously is followed by abortion in many animals and by a long-lasting immune response. A reduction of the vaccine dose induces a shorter and less intense antibody response following vaccination (Gasca *et al*., 1985; Sales-Henriques *et al*., 1992; Delgado *et al*., 1995). Accordingly, a reduced dose (10^3 - 10^6 CFU) has been used subcutaneously in field trials and reported as an effective method to control brucellosis in small ruminants and being relatively safe in pregnant sheep and goats (Kolar, 1984; Gasca *et al*., 1985; Al Khalaf *et al.,* 1992; Sales-Henriques *et al*., 1992; Kolar, 1995; Al-Shamakh, 1995; Uysal, 1995; Delgado *et al*., 1995). This method of immunisation has demonstrated its efficacy in well-controlled experiments (Gasca *et al.,* 1985). However, field trials and controlled experiments (for a review see Blasco, 1997) have demonstrated that reduced doses of Rev.1 vaccine may induce vaginal excretion and abortion in pregnant sheep and goats after field infections (Alton, 1970; Crowther *et al.,* 1977; Fensterbank *et al*., 1982, Jiménez de Bagués *et al.,* 1989). It was noted that the level of protection was poor in goats which received a dose of 10^4 CFU (Alton, 1970).

9.3. Conjunctival vaccination with Rev.1

9.3.1. In young replacement animals

When Rev.1 vaccine is administered in young replacement animals by the conjunctival route (0.5-2 x 10^9 CFU contained in a small volume of 30-50µl inoculated in the conjunctival sac), the protection conferred is similar to that induced by the classical subcutaneous method but the serological response evoked is significantly reduced. Thus, this route of vaccine administration is compatible with eradication programmes based on test and slaughter principles (Fensterbank *et al.,* 1985; Jiménez de Bagués *et al.,* 1992; Díaz-Aparicio *et al.,* 1994; Marín *et al.,* 1999).

9.3.2. In adults animals

The induction of abortion when vaccinating pregnant sheep and goats means that there is no entirely safe strategy for mass vaccination with Rev.1 vaccine. When vaccinating pregnant sheep and goats, the conjunctival method results in fewer abortions compared to the subcutaneous method. However, a significant proportion of sheep and goats vaccinated conjunctivally with standard doses excrete Rev.1 vaccine and abort (Jiménez de Bagués *et al*., 1989; Zundel *et al.,* 1992). Most of these vaccine-induced abortions take place between 40 and 60 days after vaccination, depending mainly on the stage of pregnancy at the time of vaccination. The percentage of abortions obtained when sheep are vaccinated during the last month of pregnancy is significantly lower than when vaccinated at two months of pregnancy (Jiménez de Bagués *et al*., 1989). When administered conjunctivally, a reduced Rev.1 dose induces fewer abortions than the standard dose (Jiménez de Bagüés *et al*., 1989; Zundel *et al*., 1992). However, the experimental studies that are available are inconclusive at determining the maximum dose which is compatible with an acceptable degree of safety. The minimun dose explored under controlled studies is 1 x 10^8 CFU (Zundel *et al*., 1992), and the authors concluded that its innocuousness was not yet sufficient to propose the indiscriminate vaccination of sheep and goats with this dose by the conjunctival route, whatever the age or physiological status of the animal. However, vaccination with a reduced dose of 1 x 10^6 CFU of Rev.1 by this route does not confer adequate immunity in sheep, even after revaccination (Fensterbank *et al*., 1982). Furthermore, it has been reported that the conjunctival vaccination of pregnant sheep with doses ranging from 1 x 10^6 to 1 x 10^7 CFU can be accompanied by vaccine induced abortion in vaccinated sheep (Ferrer and Gil, 1994). Therefore, as with subcutaneous vaccination, the use of reduced doses of Rev.1 administered by the conjunctival route does not fully eliminate the risk of abortion and moreover, the level of immunity conferred on sheep and goats might be insufficient.

Under experimental conditions, the minimum dose applied by the CJ route that has been demonstrated as effective against brucellosis and *Brucella* infection are: 4 x 10^8 CFU in lambs (Fensterbank *et al.,* 1985), 1.1 x 10^7 CFU in kids (Fensterbank *et al.,* 1985), and 4.5 x 10^8 CFU in ewes (Durán-

Ferrer, 1998). The minimum dose administered by the SC route that demonstrated its efficacy was 1-2 x 10^5 CFU (Gasca *et al.,* 1985).

9.4. Adverse effects of vaccination in animals

9.4.1. In young replacement animals

When Rev.1 is administered by the standard method (1-2 x 10^9 CFU subcutaneously) in young (3-6 months old) replacement animals, it induces a solid and durable immunity but also a long-lasting serological response, making the interpretation of serological tests, conducted after vaccination, difficult (Fensterbank *et al.,* 1982; Jiménez de Bagués *et al.,* 1992; Díaz-Aparicio *et al.,* 1994). This causes obvious problems for the application of combined vaccination and test and slaughter eradication programmes.

9.4.2. In adult animals

The classically recommended exclusive vaccination of young replacement animals has failed to control brucellosis in some EU countries and is frequently inapplicable in many parts of the world. Accordingly, the whole-flock vaccination appears the only feasible alternative to control *B. melitensis* infection in small ruminants in endemic situations and also under the extensive management practices characteristic of some EU Mediterranean countries. When used exhaustively in high prevalence situations, the Rev.1 vaccine reduces the transmission process and, in the medium term, leads to a substantial decrease in the prevalence of brucellosis in both small ruminants and humans (Elberg, 1981 and 1996), allowing then the application of test and slaughter policies for the definitive eradication of the disease.

However, the Rev.1 mass vaccination strategy has two main drawbacks:

i) the vaccination of pregnant animals with standard Rev.1 doses administered subcutaneously is followed by vaccine induced abortion in many animals (Alton and Elberg 1967; Elberg, 1981; Jiménez de Bagués *et al.,* 1989; Zundel *et al.,* 1992; Blasco, 1997).It has been stated that the capability of the Rev.1 strain to induce abortion is a phenomenon that depends on dose and on time of pregnancy when the females are vaccinated: a reduction of the dose increases the degree of safety, and the risk of abortion is minimized when animals are vaccinated at the end of pregnancy or during lactation (Jiménez de Bagüés *et al*., 1989; Elberg, 1996).

ii) the vaccination of adult animals with standard Rev.1 doses administered subcutaneously induces a long-lasting serological response, making it difficult to discriminate the serological response evoked, when test-and-slaughter eradication programmes are simultaneously operated (Jiménez de Bagués *et al.,* 1992; Marín *et al.,* 1999). In general, the residual immune response induced by the vaccination is of less intensity and duration when the dose is reduced and/or the conjunctival route is used.

9.5. Handling Rev.1 vaccine and its use in the field

a) Zoonotic aspects

The Rev.1 vaccine strain can cause infection in humans (Blasco and Díaz 1993) and should therefore be handled and used with care.

b) Quality control

Until recently, quality control of Rev.1 vaccine included only *in vitro* criteria such as the absence of contamination, adequate viable counts and the assessment of the typical colonial morphology. However, it has been demonstrated on purified strains from phase-dissociated Rev.1 commercial vaccines (Bosseray, 1991) that these requirements alone do not guarantee the immunological quality of the vaccines. For a proper quality control, *in vitro* criteria should be complemented with the assessment of the biological proprieties of vaccines through the study in target species (OIE Manual, 1996) as well as in guinea pigs (Alton and Elberg, 1967; WHO, 1977) or mice. In mice, both residual virulence (Recovery Time 50%, RT50) and immunogenicity tests have been developed (Plommet and Bosseray, 1977, 1984; Bosseray *et al.,* 1984; Bosseray, 1985,1991,1992a,b; Garin-Bastuji, 1992). The mouse model has been recently recommended by the OIE as a reference method for the quality control of Rev.1 and B19 vaccines (OIE Manual, 1996).

Residual virulence of the vaccinal strain is a key point in the quality of the Rev.1 vaccine. In the late 1980s, a virulent giant colonies (G) forming mutant ("FSA" mutant) of the Rev.1 vaccine was detected in South Africa (Pefanis *et al.,* 1988; Pieterson *et al*., 1988; Hunter *et al.,* 1989). This was responsible for abortion, orchitis, and other clinical signs in inoculated small ruminants. The strain also showed slower clearance in guinea pigs and mice when compared with the standard Rev.1 strain, and horizontal transmission was achieved in 2 sheep (Hunter *et al.,* 1989).

When tested in the mouse model, differences in residual virulence and immunogenicity have been demonstrated between various Rev.1 vaccines produced world-wide. These differences could explain the contradictory safety results of field trials using reduced doses of Rev.1 vaccine. Bosseray (1991) compared five commercial *B. melitensis* Rev.1 vaccines from different sources with a standard Rev.1 strain. After purification, one smooth strain out of five had the same activities as the standard strain and three were as immunogenic but less virulent. In fact, it has been documented (Blasco, 1997) that a commercial Rev.1 vaccine that did not induce abortions in pregnant sheep in field trials carried out with reduced doses in Spain (Blasco *et al*., 1984) and Portugal (Sales-Henriques *et al*., 1992), showed low residual virulence when tested in the mouse model (Bosseray, 1991). On the other hand, Zundel *et al.* (1992), after a study on safety of the standard Rev.1 vaccine, concluded that its innocuousness was not yet sufficient to propose a dose of 1×10^9 CFU to indiscriminately vaccinate sheep and goats by the conjunctival route, whatever the age or physiological status.

Finally, it was agreed (WHO, 1997) that "correctly standardised Elberg 101 strain Rev.1 vaccine should continue to be considered as the basis of brucellosis control in small ruminants where vaccination is applied, until new safer and effective versions of *B. abortus and B. melitensis* vaccines, based on rough strains, have been tested under controlled experimental and field conditions and shown to be at least equivalent to the Rev.1 vaccine". The guidelines for the correct standardisation of the Rev.1 strain are given in the European Pharmacopeia (1997).

c) Use in young animals

In the case of implementing a Rev.1 vaccination (young replacements) programme combined with a further test and slaughter strategy based on serological tests such as the RB and CF tests, as is currently the case in some EU countries, the use of conjunctival vaccination should be considered. However, there is currently only limited availability of Rev.1 vaccines suitable for conjunctival application.

d) Use in adult animals

Conjunctival vaccination with Rev.1 is safer than subcutaneous vaccination, but the conjunctival method is still not safe enough to be applied regardless of the pregnancy status of the ewes and should therefore be used only under strictly controlled conditions (Jiménez de Bagués *et al.,* 1989; Zundel *et al.,* 1992). When the objective of a mass vaccination campaign is the induction of a high level of immunity in the population, with minimal adverse effects, the conjunctival administration of standard doses of Rev.1 during the late lambing season - lactation - prebreeding period could be recommended as the most efficient whole-flock vaccination strategy (Blasco, 1997).

9.6. Other recently developed vaccines

B. suis S2

B. suis S2, a classically obtained Brucella attenuated strain with smooth LPS, was apparently successfully used in controlling brucellosis following oral administration to small ruminants in field conditions in China (Xin, 1986) and Libya (Mustafa and Abusowa, 1993). However, this vaccine showed no protective effect against B. melitensis in sheep in fully controlled experimental conditions (Verger *et al.,* 1995).

B. abortus RB51

Another classically obtained, live attenuated but rough (S-LPS lacking) Brucella strain is the B. abortus RB51 strain (Schurig *et al.,* 1991). The strain RB51 has been reported to be equally effective as S19 vaccine in protecting against B. abortus in cattle, without inducing anti-O chain antibodies as detected by serological tests (Palmer *et al.*, 1997). Preliminary experiments suggest that this vaccine can be effective for the prophylaxis of B. melitensis infection in goats (Suarez *et al.,* 1998). In contrast, it has been demonstrated that this vaccine does not confer protection against B. ovis in controlled experiments in rams (Jiménez de Bagués *et al.,* 1995).

VTRM1

VTRM1 is a live rough strain obtained by transposon mutagenesis from smooth B. melitensis 16M. Mutagenesis resulted in a truncated gene for mannosyltransferase, an enzyme necessary for the synthesis of the S-LPS and accordingly, VTRM1 does not induce antibodies against the S-LPS and does not interfere with classical serological tests. However, the VTRM1 vaccine does not confer adequate protection against B. melitensis in goats (Elzer *et al.,* 1998).

RfbK strain

The mutant rfbK is a live rough strain obtained by transposon mutagenesis from smooth B. abortus 2308 (Adams *et al.,* 1998). Mutagenesis resulted also in a truncated gene for mannosyltransferase. Preliminary experiments suggest that this mutant strain can be effective for the prophylaxis of B. melitensis infection in goats (Suarez *et al.,* 1998).

Novel vaccines

Other interesting approaches to develop new generation vaccines, such as the construction of recombinant strains deleted in relevant diagnostic proteins, or DNA based vaccines, are being currently investigated. It is probable that some of these vaccines will become candidates to replace the available classical vaccines in the near future. However, to date, none of these vaccines have been found to surpass the immunity conferred by the classical Rev.1 vaccine.

Accordingly, until new and more effective vaccines have been developed and tested properly under controlled conditions, the Rev.1 strain should continue to be the reference vaccine for the prophylaxis of brucellosis in small ruminants.

9.7. Conclusions

There is agreement that vaccination of sheep and goats is the only practical and effective procedure to reduce the prevalence of brucellosis in many countries. In most developing countries and in some EU Member States affected by *B. melitensis*, vaccination of sheep and goats continues to be indispensable to control the disease. The live attenuated *B. melitensis* Rev.1 (Elberg 101) strain is the best classical vaccine available for prophylaxis of brucellosis in small ruminants. Its use is recommended by WHO (1997), and it has its own monograph in the EP. Vaccine Rev.1 confers a high degree of protection but cannot prevent a transitory wild-type infection. Thus, the effect of vaccination is quantitative, resulting in reduced and shortened excretion of Brucella in case of infection and hence in a reduced disease transmission and contamination of the surroundings. Vaccination is effective only if applied according to a strict control programme. Rev.1 displays residual virulence and can induce abortions when applied improperly. The vaccine is administered by the sc or the conjunctival (cj) route. Numerous experiments have been carried out to optimise the vaccination scheme and to minimize adverse effects of the vaccination. Vaccinated animals produce an

antibody response that can not be differentiated from that after infection with the wild-type Brucella, which influences control programmes based on serological tests with a view to eradicate the infection in a certain area. The degree of immunity or resistance to infection depends on the dose of bacteria contained in a vaccine dose and on the route of vaccination. When administered in pregnant sheep and goats, cj vaccination may reduce the rate of abortions following vaccination. Rev.1 vaccine can cause infection in humans and must be handled with care. Control of safety and efficacy is described in the OIE Manual and the EP. Production of the vaccine follows a seed-lot system and requires continuous verification of the properties of the strain. Deviation from the original strain has been observed.

Of other vaccines, the *B. suis* strain S2 appears not to be protective in sheep. The rough B. abortus strain RB51 may be effective in goats against *B. melitensis* infection but failed to protect from B. ovis infection. It does not induce antibodies against S-LPS. VTRM1 is a rough *B. melitensis* strain and does not interfere with antibodies to S-LPS, but failed to confer adequate protection in goats. The rough mutant rfbK of B. abortus may be effective in protecting goats.

10. STRATEGIES FOR THE CONTROL AND ERADICATION OF BRUCELLOSIS IN SMALL RUMINANTS

Because brucellosis is a disease of major economic and zoonotic importance, a strategy for its control in small ruminants is essential in endemic areas. The initial aim of the strategy selected will be the reduction of infection in the animal population to such a level that the impact of the disease on human health as well as on animal health and production will be minimised (Kaplan, 1966). Subsequent steps can include eradication from a region by test and slaughter and, following successful eradication, measures to prevent reintroduction of the disease.

Control of a zoonosis is a general term that embraces all the measures designed to reduce the incidence and prevalence of a disease in a defined animal population.

The term eradication has first been used to mean the extinction of an infectious agent. Accordingly, eradication is not completed if a single infectious agent survives anywhere in nature (Cockburn, 1963). The meaning of the term has been modified with time and the most common meaning of eradication in veterinary practice refers to the regional extinction of an infectious agent.

The term elimination has been created to identify an intermediate situation. Elimination refers to the reduction in the incidence of the disease below the level achieved by control, so that either very few or no cases occur, although the infectious agent may persist (Payne, 1963; Spinu and Biberi-Moroianu, 1969). Elimination is the task for most programs implemented for the control of brucellosis in small ruminants.

It is accepted that before a zoonosis control program is designed and implemented, a well functioning surveillance system, which should be fed with valid data collected from the field, must be established. The main purpose of the surveillance system will be to determine the prevalence of the disease, so that appropriate measures for its control can be taken. The system must be able to detect early any change in incidence and prevalence. The surveillance system allows for the optimisation of resources, for alternative actions to be designed, for the continuous justification of expenses, and the evaluation of activities performed and achievements to be assessed (Thrusfield 1995: WHO/MZCP 1998).

Once an accurate surveillance system is in existence and is fed with valid data collected from the field, the progress, impact, adequacy, efficiency, and efficacy of a control program can be continuously assessed and evaluated.

It is a crucial matter that the information output must be issued not only to the decision-makers but also to all the members participating in the program, so that they can be informed about the progress of their work. This is very important especially in zoonosis control programs, because the advantages of the program are not so obvious as in other diseases, whose occurrence is characterised by heavy losses in animal population. There are many cases that zoonosis control programs had failed because the people participating had lost motivation since they did not see any obvious benefit (WHO/MZCP 1998).

10.1. Possible strategies

A selection can be made from the following strategies, which are implemented for the control of brucellosis.

1. Immunisation to reduce the rate of infection in specified herds.

Control of brucellosis can be achieved by using vaccination to increase the population's resistance to the disease. Vaccination practically eliminates the clinical signs of brucellosis and is accompanied by a reduced contamination of the environment as well as exposure of the population at risk to the infectious agent (Nicoletti, 1993).

The initial campaigns to control brucellosis in small ruminants were based on the vaccination of young animals, kept as replacements, with the Rev.1 vaccine. This approach was based on the hypothesis that the Rev.1 vaccine offered life-long immunity and that after implementing the vaccination program for 5-7 years (the productive life-span of sheep and goats) the whole population would be vaccinated and fully protected against brucellosis. This method was also recommended to minimise postvaccinal diagnostic problems and to avoid abortions (Nicoletti, 1993; Blasco, 1997).

However, in many countries, where the animals were kept under extensive conditions with nomadic or semi-nomadic husbandry, this approach was impractical and failed to reduce the incidence and prevalence of the disease, because the development of herd immunity was very slow (Kolar, 1995; Blasco, 1997). In addition, the unvaccinated adult animals remain unprotected and the infection can spread (Kolar, 1995).

Vaccination of all animals (young and adults) in a flock or region is an alternative approach for the control of brucellosis in small ruminants. Mass immunisation of small ruminants has been applied with success in many countries under different socio-economic conditions. In general, mass immunisation is indicated where the prevalence of infected animals is high. It should be based on knowledge of the prevalence in the flock or region.

Mass vaccination of a flock helps to rapidly establish a relatively immune stock, and reduces the level of abortions and excretors of *Brucella*, thus reducing contamination of the environment and disease transmission (Kolar, 1995).

However, this strategy has the limitation that pregnant animals can not be vaccinated because the vaccine is not innocuous enough for pregnant animals, and the efficacy of the strategy depends on the continuous availability of the vaccine (WHO, 1986).

Provided that the prevalence of disease is moderate, financial resources are available, and a well functioning surveillance by the veterinary service is in place, vaccination of young animals can be combined with a test and slaughter policy in a long term action to control brucellosis in small ruminants (WHO, 1998).

2. Elimination of infected animals by test-and-slaughter to obtain brucellosis free flocks/herds and regions.

It is usually accepted that a programme of eliminating brucellosis by test-and-slaughter policy is justified on economic grounds only when the prevalence of infected animals in an area is about 2% or less (Nicoletti, 1993).

For the implementation of such a program it is essential that the flocks are under strict surveillance and movement control. Animals must be individually identified and an efficient and well organised veterinary service for surveillance and laboratory testing must be in place (Alton, 1989; Nicoletti, 1993).

The flock size as well as the prevalence of brucellosis are the most important factors of this strategy which has been shown to be ineffective and unreliable when attempted in large flocks with a high prevalence of brucellosis (Kolar, 1995). The limited reliability of the diagnostic tests used which are unable to reveal all infected animals and which may give false negative results due to incubation period, latency or due to criteria used to interpret the results must also be considered. These drawbacks apply more to sheep and goat flocks than to cattle herds where a test and slaughter strategy has been more effective (Kolar, 1984; Nicoletti, 1993).

Before embarking on the implementation of such a strategy it is necessary to ensure that the epidemiological situation is favourable, the necessary facilities and financial resources are available, a pool of healthy replacement animals is available and that the resources exist for continuing surveillance for a considerable period. It is also essential that full co-operation of farmers is available as slaughter of seropositive animals can be resisted by owners because of lack of clinical signs, inadequate compensation or lack of replacement animals (Nicoletti, 1993).

A brucellosis control and eradication plan based on test and slaughter strategy can be either voluntary or compulsory. Voluntary schemes, which apply to individual flocks, may be useful in the early stages of the campaign but may need to be supported by adequate incentives such as a bonus on the sale of milk from brucellosis-free herds or per capita payments. Compulsory eradication is required in the final stages but is often advisable from the start (WHO, 1986).

3. Prevention of spread between animals and monitoring of brucellosis-free herds and zones.

In areas where the brucellosis-free status has been established or where such a status is suspected on epidemiological grounds, the risk of importing the disease by means of animal movement must be curtailed. Movement of potentially infected animals into such areas must be prohibited or importation permitted only from certified brucellosis-free farms or areas. This applies to the transport of animals and of certain animal products within countries as well as between countries, following the general principles and procedures as specified in the International Zoo-sanitary Code of the OIE

which also describes the essential testing of animals and quarantine measures.

It should be noted that there have been incidents of spread of brucellosis by serologically negative animals originating from inadequately certified and supervised sources (Schnurrenberger *et al.,* 1987; WHO, 1986).

10.2. Strategy selection

Different countries and even ecologically distinct areas within a country may require different strategies for the prevention and control of brucellosis in the population of small ruminants, depending on epidemiological and socio-economic conditions.

Decisions as to the appropriate strategy for the control and/or elimination of brucellosis are usually a national responsibility, though in some large countries this may be delegated to regions or provinces or made applicable to individual islands or communities.

A selection may be made from the strategies described with the understanding that they are not necessarily mutually exclusive. In deciding a strategy, many factors including the following must be considered: the type of animal husbandry, the geography of the area, the patterns of commerce, financial, technical and personnel resources available and, most importantly, the prevalence of disease and the acceptance of strategy by the livestock owners (WHO/MZCP 1988).

In addition to the strategy selected, simple non-specific measures of prevention of the spread of the infection should be applied in a systematic way. These include general hygiene measures and the provision of clean accommodation that can be disinfected at parturition. All parturitions in an infected flock should be regarded as potential sources of infection and all the non-living products and contaminated materials must be incinerated or buried (Alton, 1990).

The provision of information and education concerning the disease to farmers and local communities is essential. The farmer must be informed of all the advantages of the control campaign , such as the economic benefits and the elimination of risk to health of his family and himself.

Professional training is essential for the implementation of the strategies by the appropriate national services. The training of the different groups in society will ensure that the right actions are taken and that the necessary resources are mobilised (WHO, 1986).

The main points which must be taken into account for the choice of the appropriate strategy for the control of brucellosis are illustrated in the following diagram (Fig. 1).

Figure 1. Decision Chart For Brucellosis Control In Animals (WHO / MZCC, 1998)

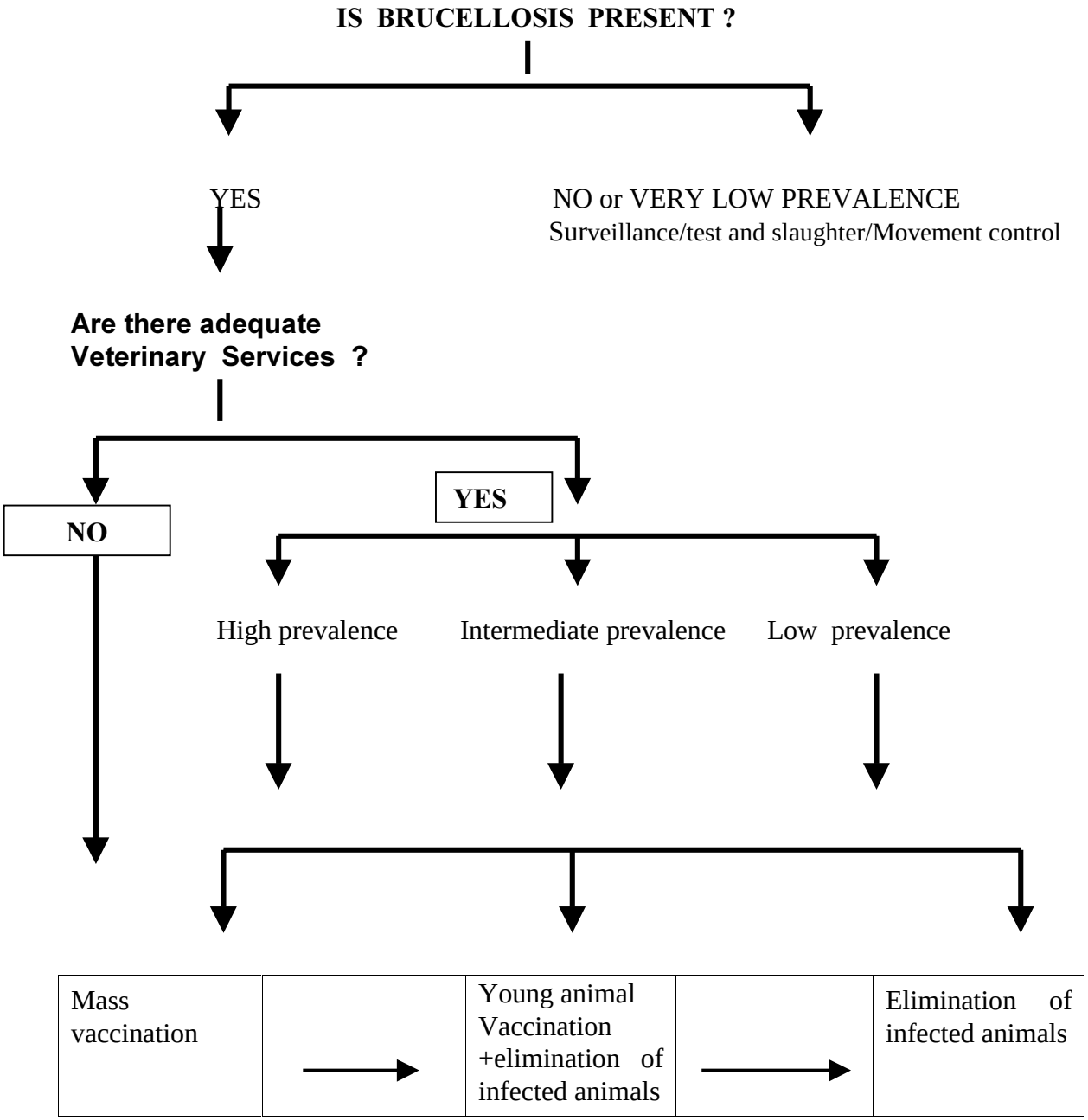

Table 8: Summary of the advantages and disadvantages of various strategies

Advantages and Disadvantages of Brucellosis Control Strategies		
STRATEGY	ADVANTAGES	DISADVANTAGES
Mass vaccination	Reduces zoonotic impact Herd immunity quickly established Effective disease control and reduction in losses due to disease Well accepted by owners Easy to manage and economical Flock immunity can be maintained by vaccinating young animals.	Vaccine induced abortions in pregnant animals Distinguishing infected from vaccinated animals is not feasible in the short term Infected animals remain on farms for some time.
Vaccination of young animals and test and slaughter of older infected animals	Minimises vaccine induced abortions Serological response reduced in vaccinated non-infected animals allowing test to differentiate infected and vaccinated animals	Herd immunity slowly established (unless moving from mass vaccination strategy) Serological tests to differentiate infected and vaccinated animals are not optimal and cannot be relied upon for accurate diagnosis of an individual animal
No vaccination Test and slaughter	If successful will result in elimination of the infection in the region. Diagnostic tests are more accurate in non vaccinated animals but still not optimum.	Risk of epidemics and subsequent human infection Higher cost Need efficient veterinary services (animal identification, laboratory support, movement control) Suitable for low disease prevalence areas only Removal of protective cover of vaccination may allow disease prevalence to increase May require whole herd slaughter to be effective

Although it is not possible to suggest precise recommendations which are appropriate for all conditions and countries it is important to define high, intermediate and low prevalence of the disease. In general, if the prevalence in the small ruminant population exceeds 5% of the animals only systematic vaccination is recommended. If the prevalence is between 1% and 5% of the animals a program which combines vaccination of young animals and test and slaughter policy can be considered, if adequate financial resources are available. Where the seroprevalence is below 2% (preferably 1%) of the flocks and herds in an epidemiologically isolated area then a short-term program based only on a test and slaughter policy may be implemented (MZCC, 1986; Nicoletti, 1993).

It should be kept in mind that the serodiagnostic techniques currently used (RBT and CFT) are less sensitive in small ruminants infected with *B. melitensis* than in *B. abortus* infected bovines (Farina, 1985). Therefore the proportion of *B. melitensis* infected small ruminants which go undetected is higher (Garrido, 1992).

The decision to cease vaccination with Rev.1 when the serological prevalence is low, and to move to a test and slaughter strategy should be taken only after successfully completing a pilot project in a restricted area, under large-scale field conditions. (Garrido, 1992).

Experiences of the withdrawal of the Rev.1 vaccination in endemically infected small ruminants populations, have been relatively poor. Authorities contemplating this step should therefore proceed with caution and carry out appropriate risk assessment and pilot projects.

Computer modelling may be of value in planning the detailed strategy of control and eradication programmes predicting how some variables, such as herd size, management systems, breeding patterns, immunisation status, and other factors, can influence the choice of the most effective course of action. Predictive modelling is considered as being complementary to practical field knowledge rather than substituting it. Techniques are available for cost benefit analysis in brucellosis and may be of considerable assistance in deciding the best approach to its control under a given set of circumstances.

Eradication of an infection implies the disappearance of the infectious agent from a given area. A highly organised effort is needed to reach eradication in either an area or in a population.

On a national scale, eradication has so far been achieved only by identifying infected herds and slaughtering all the animals in the herd. This procedure is normally only feasible where *B. melitensis* infection has been newly introduced into a previously non-infected region or where the prevalence is very low. (WHO, 1986)

If brucellosis is well established in a defined area, eradication is more difficult and requires resources over a considerable period. The technical tools available have been shown to be adequate to achieve this objective only

under certain circumstances. For example, the eradication of *B. melitensis* from goat herds by test-and-slaughter has been shown to be feasible in a trial in 50 herds in Malta using the complement-fixation test as diagnostic test. The feasibility of eradication by test-and-slaughter in small ruminants appears to depend largely on the conditions under which animals are kept. The chances of success are greatest in small isolated flocks kept under close control and least in large flocks, especially when they can mix with other flocks and/or are herded in large numbers in enclosures at night. The risks involved in the system of transhumance also need special attention (WHO, 1986).

Only one substantial area, part of Cyprus, has successfully eradicated brucellosis in small ruminants after it was considered to be of significant prevalence (Nicoletti, 1993). The Balearic Islands have arrived to 0.00% prevalence in the 1999 eradication campaign from a previous 0.68 % in 1994 (M.A.P.A., 2000).

Taken together, where the overall individual prevalence of the disease is below 2% and where flocks are maintained under closely controlled conditions and can be protected against re-infection, eradication is feasible, on a farm or regional basis, provided:

(a) an adequate veterinary organisation for surveillance and laboratory testing is available,

(b) the administrative means and willingness to implement the programme, including the control of movements of animals and adequate financing are present, and

(c) co-operation of the animal owners with the programme exists.

Where *B. melitensis* infection is endemic and widespread in the population of small ruminants, control by immunisation is recommended, at least as a preliminary step in most situations. For the eradication of brucellosis in small ruminants the strategies described must be implemented in combination. Such steps can be arranged in a cascade (see Fig. 1 and Fig. 2) indicating that eradication can be achieved in a long-term programme.

10.3. Conclusions

Brucellosis in sheep and goats is an important zoonosis and from this aspect alone requires control programmes aimed at the final eradication of the disease in affected regions. There exists a positive correlation between the level of *B. melitensis* infections in small ruminants and the number of infections in humans. Control of the disease in animals is a prerequisite to reduce its zoonotic spread. Control of infection in animal populations can be based on different strategies the selection of which depends on numerous factors. Mass vaccination accompanied by a strict surveillance scheme is a first step to reduce the number of infected animals and hence the infection pressure. At a low level of infection a test-and-slaughter programme can be applied in order to attain brucellosis free flocks and zones. The creation of brucellosis-free flocks and zones is a second level, which requires that re-

infection is prevented and that young replacement animals are available in a sufficient number. Application of a control and finally eradication programme can be negatively affected by traditional factors of husbandry of small ruminants, in particular mixing of flocks in transhumance areas and movement of flocks to different places in the course of a year. Eradication programmes require constant surveillance and financial support from state authorities. Socio-economic factors play an important role in eradication programmes.

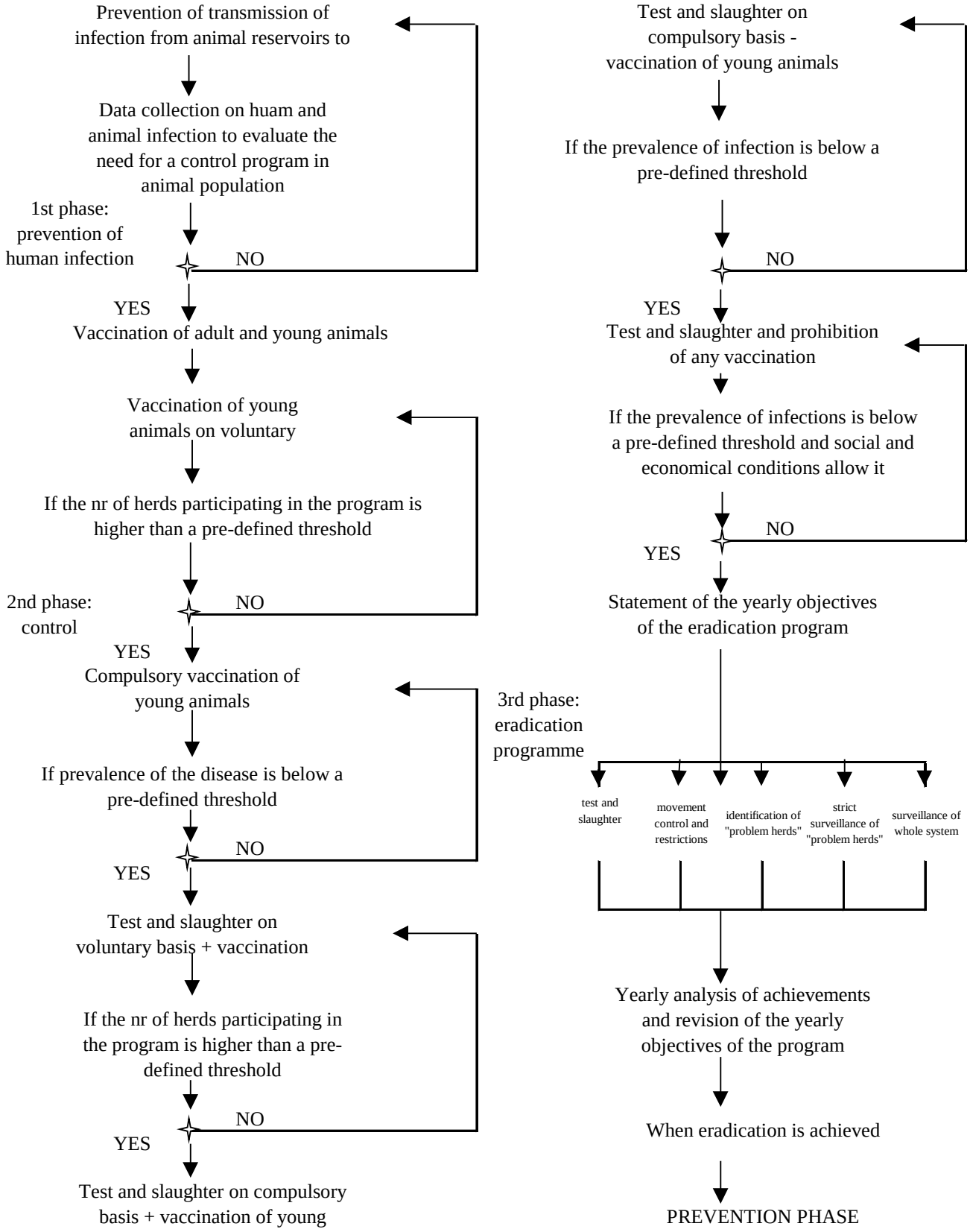

Figure 2. Phased control and eradication programmes

11. FUTURE RESEARCH

Four areas on which future research is considered useful and necessary are identified in the Report:

1. Bacteriology of the *Brucella* species

Taxonomic knowledge of *Brucella* has largely been improved with the introduction of techniques of molecular biology and genetics. More has to be done to harmonise methods of molecular identification and typing of *Brucella.* Whereas *B. melitensis* biovar 3 appears to be the most frequently isolated species in the Mediterranean countries its differentiation from biovar 2 is complicated and sometimes equivocal. Generation of standardised monospecific sera or monoclonal antibodies is required. Phenotypic characteristics used for classification of *Brucella* show a certain degree of instability leading to so-called intermediate strains. This situation impedes the identification of the species and their biovars. Therefore, the identification of stable DNA-specific markers should be given a high priority for taxonomic, diagnostic and epidemiological purposes. PCR-RFLP and Southern blot analysis of various genes or loci can been employed to find DNA polymorphism. Research in this field could provide useful tools to enable molecular identification and typing of the *Brucella* species and their biovars. Extension of knowledge on the bacterium will lead to improved vaccines and improved diagnostics.

2. Diagnosis and differential diagnosis of *Brucella* infections in animals

In view of the practical importance of *Brucella* infections classical and molecular-biological techniques should be investigated to improve the bacteriological diagnosis. This includes the development of selective media which are more efficient in isolating all *Brucella* species. PCR techniques should be further developed to enable the detection of *Brucella* specific DNA fragments in samples of poor quality from the field.

The nature and characteristics of a universal antigen for the diagnosis of smooth *Brucella* strains is not generally agreed. This question is directly related to the problem of which *Brucella* species and biovars should be used for the production of diagnostic antigens for samples from sheep and goats. The Rose Bengal (RB) test and the complement fixation (CF) test are the most widely used tests for the serological diagnosis of sheep brucellosis. The antigenic suspensions (whole cells) used in both tests are made with *B. abortus*. The RB antigen used for the diagnosis in sheep and goats should be standardised specifically for this purpose and antigen standardised for use in cattle should not be used.

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 sera from *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. 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. 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. This type of antigen should be investigated for use in ELISA. Improved techniques could show if these antigens are able to differentiate Rev.1 vaccinated from *B. melitensis* infected ewes.

Further research is needed on the identification, isolation, characterisation and cloning of both inner and outer membrane proteins which could be used as diagnostic antigens. This should be followed by the development of subunit or live antigen-deleted vaccines, able to protect animals without interfering with diagnostic tests. While the use of ELISA for the diagnosis of bovine brucellosis is well developed, more extensive field trials are required to fully validate the test for use in sheep and goats.

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

Available information on the application of the gamma-interferon test for the indirect diagnosis of *Brucella* infection is scarce. Research is needed for the accurate estimation of performance characteristics of the test (sensitivity, specificity, repeatability and reproducibility) and its usefulness under various epidemiological conditions.

The use of bulk milk or sera samples for serological testing would be an advance, allowing in particular milking sheep flocks to be tested on a regular and frequent basis. Research is required to determine if a suitable test exists for this purpose.

3. Cross reactions of infections by *Brucella* species

Where *B. ovis* and *B. melitensis* co-exist in sheep populations, the question of cross-reactions is important. There is little information how smooth and rough *Brucella* antigens affect the routine diagnosis of *B. melitensis* infection. The smooth *Brucella* antigens used in classical tests depend on S-LPS and should not react with antibody against the rough *B. ovis*. However, in iELISA tests preliminary reports suggest that extensive cross-reaction could occur. The interference of other microbial agents like *Yersinia enterocolitica* deserves further attention with a view to develop discriminating tests. The competition ELISA may be a useful technique to achieve this goal.

4. Vaccine research and optimisation of vaccination schemes as part of control programmes

Advantages and disadvantages of vaccination of small ruminants with the classical Rev.1 vaccine have been investigated in detail. Research is necessary to determine if other and more modern vaccines will be able to avoid or overcome the problems. The Report mentions a few vaccines recently developed from *B. abortus*, *B. suis*, and *B. melitensis* strains, whereby techniques of molecular biology have been applied. Other new generation vaccines, such as the construction of recombinant strains deleted in relevant diagnostic proteins, or DNA based vaccines, are currently

being investigated and deserve support. It is probable that one of these vaccines will become a candidate to replace the classical available vaccines.

The role of genetics and breed effects in susceptibility to infection with *Brucella melitensis* could be investigated.

12. SUMMARY AND GENERAL CONCLUSIONS

Brucellosis caused by infection with *B. melitensis* is an important zoonosis. Human brucellosis is widely distributed all over the world, with regions of high prevalence such as the Mediterranean region, Middle East, Latin America and Asia. The incidence in humans can vary widely between different regions, with values of up to 0.2%. Besides this potential, brucellosis in sheep and goats is an important animal disease which affects many regions where small ruminants are the predominant species of domestic animals. It is therefore essential to have control programmes in place aimed at prevention of spread of the infectious agent to humans and to eradicate the disease.

In sheep and goats, the major clinical sign is abortion but other signs may be observed, such as orchitis, epididymitis, hygroma, arthritis, metritis, subclinical mastitis. Animals may develop self-limiting infections or become latent carriers with potential excretion of the bacteria. This stage is frequently associated with a persistent infection of the mammary gland and supramammary and genital lymph nodes with shedding of the organisms in milk and genital secretions. Abortion generally does not occur if the female becomes infected towards the end of pregnancy.

The Rose Bengal (RB) plate agglutination test and the complement fixation (CF) test are the most widely applied tests for the serological diagnosis of a *B. melitensis* infection in small ruminants. The ELISA will probably be the test of choice in the future. These tests have been developed for the diagnosis of *B. abortus* infections in cattle. None of the tests has been fully validated for use in sheep and goats. These tests and any developed in the future should be validated according to OIE standards. ELISAs and native hapten precipitation tests have been developed which could be useful for differentiating *B. melitensis* infected from Rev.1 vaccinated sheep, but extensive experience is lacking. Other tests are the antiglobulin (Coombs) test and the Fluorescence Polarisation Assay (FPA). These have so far not reached a wide application in the diagnosis of *B. melitensis* infections. A Community Reference Laboratory and a network of National Reference Laboratories is required to co-ordinate activities and to ensure international harmonisation of tests and procedures.

Tests based on cell-mediated immunity are the Brucellin test and the gamma-interferon test. Whereas the Brucellin test is standardised and used in cattle testing, the gamma-interferon test requires further research.

The live *B. melitensis* Rev.1 vaccine is a useful tool to control brucellosis in sheep and goats and to decrease infections with *B. melitensis* in humans. In whole flock vaccination programmes it greatly decreases the prevalence of brucellosis in both sheep and human populations. It requires proper administration in relation to the epidemiological situation. 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 test-and-slaughter of adult animals. At a final stage, it may be possible to use a test-and-slaughter programme only.

The major drawback of the Rev.1 vaccine is that its degree of attenuation is insufficient to allow its use without restrictions. Its residual virulence may induce abortions and also lead to persistent serological responses as indicated by the classical methods of serological diagnosis. This will inevitably interfere with an eradication programme based on a test-and-slaughter policy. In order to minimise these adverse effects, results of studies on different procedures for the administration of the vaccine (conjunctival route instead of subcutaneous route and/or reduction of the dose of vaccine) have been conducted. In general, it was demonstrated in these studies that less adverse effects were noted when the vaccine was applied by the conjunctival route.

A WHO paper (1997) states that correctly standardised Elberg 101 strain Rev.1 vaccine should continue to be considered as the basis of brucellosis control in small ruminants where vaccination is applied, until new safer and effective versions of *B. abortus* or *B. melitensis* vaccines have been tested under controlled experimental and field conditions and shown to be at least equivalent to the Rev.1 vaccine.

Because brucellosis is a disease of major economic and public health importance, a strategy for its control in small ruminants is essential in endemic areas. The initial aim of the strategy selected will be the reduction of infection in the animal population to an acceptable level so that the impact of the disease on human health as well as on animal health and production will be minimised. Subsequent steps must include eradication from a region by test and slaughter and, following successful eradication, measures to prevent reintroduction of the disease.

It is usually accepted that a programme of eliminating brucellosis by a test and slaughter policy is justified on economic grounds only when the prevalence of infected animals in an area is about 2% or less. In areas where the brucellosis-free status has been established or where such a status is suspected on epidemiological grounds, the risk of importing the disease by means of animal movement must be curtailed. Movement of potentially infected animals into such areas must be prohibited. Importation may be permitted only from certified brucellosis-free farms or areas.

Experience on the cessation of the Rev.1 vaccination in endemically infected small ruminant populations is relatively poor. Authorities contemplating this step should proceed carefully and carry out appropriate risk assessment and pilot projects.

13. RECOMMENDATIONS

The Scientific Committee on Animal Health and Animal Welfare recommends that:

(1) tests for the diagnosis of *B. melitensis* be developed which enable the differentiation between vaccinated and infected sheep and goats. Tests should be validated and suitable for automation. They should exhibit a sensitivity and specificity at least equivalent to tests currently used. Diagnostic tests should be validated in accordance with OIE standards.

(2) efforts be made to develop a new vaccine against brucellosis in sheep and goats based on rough strains which is devoid of the disadvantages of the Rev.1 vaccine (e.g. abortions in animals, virulence for humans) while conferring a well established protection against field infections. It would be desirable to develop a vaccine which facilitates the differentiation between vaccinated and infected animals in accordance with the aims established by WHO.

(3) eradication programmes be enforced in order to protect human health. These should include in affected regions the careful identification of the animals, a full compensation policy, and movement and trade regulations to increase the probability of being successful. The strategy to be applied should be adapted to the particular situation in a country or region as described in Chapter 10.

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