Chapter 13

Control of Animal Brucellosis — The Most Effective Tool to Prevent Human Brucellosis

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Additional information is available at the end of the chapter

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Abstract

The World Health Organization classifies brucellosis as one of the seven neglected endemic zoonosis which contribute to the perpetuation of poverty in developing countries. Although most of the developed countries are free from this important zoonosis, brucellosis has still a widespread distribution in the Mediterranean region, the Middle East, Central Asia, and parts of Latin America, making it a global problem. Nearly half a million of new cases of human brucellosis are reported each year around the world, in which animals (or products of animal origin) are the most likely source of infection. *Brucella melitensis*, the main etiologic agent of small ruminant brucellosis, is the most prevalent specie involved in cases of human disease in most parts of the world. Additionally, *Brucella abortus* (main responsible of bovine brucellosis) and *Brucella suis* (the most common etiological agent of porcine brucellosis) are often associated with human brucellosis. In animal production, brucellosis has a strong economic impact due not only to its direct consequences (e.g., reproductive failures) but also to indirect loses (e.g., trade restrictions). The problem of brucellosis could be considered a clear example of the need for a "One World, One Health" strategy, given that the only approach to achieve its control and subsequent eradication is the cooperation between public and animal health authorities. The prevention of human brucellosis cannot be achieved without the control of the disease in the animals, as exemplified by the impact that the early measures adopted in the beginning of the 20th century forbidding the consumption of goat milk had on the prevalence of the disease in the British soldiers in Malta. When the prevalence of the disease in the animal population is high or when eradication cannot be achieved due to other factors (e.g., lack of economic resources), its control in livestock must be the first objective. When deciding the optimal approach to tackle the disease, the prevalence of animal brucellosis is not the only parameter to consider by the decision makers since other epidemiological and economic aspects should be considered in order to implement the most adequate control strategy in each region. Cooperation between all stakeholders involved is a cornerstone in the success of any control strategy. Strict biosafety and management measures, vaccination, and test-and-slaughter strategy are recognized as the most ef-

fective strategies to control this pathology in livestock. The adequate combination of these measures depends on several factors that will determine the success of the eradication efforts. The present chapter will review the abovementioned measures for the control and eradication of brucellosis in livestock, focusing on the advantages and drawbacks of the diagnosis tools and immunization strategies currently available and evaluating new approaches based on the advance on the knowledge of different aspects of this disease and its etiological agents.

1. Introduction

Animal brucellosis is one of the most important challenges faced by animal health authorities and producers worldwide due to the large number of host species that can be affected, limitations of the currently available diagnostic and prophylactic tools, and complex epidemiology. Yet, control and eventual eradication of animal brucellosis is the only way to ultimately win the battle against human brucellosis. Although the present chapter is focused on the current approaches for the control of *B. melitensis* in small ruminants (small ruminant brucellosis, SRB), *B. abortus* in cattle (bovine brucellosis, BB), and *B. suis* (biovars 1, 2, and 3) in swine (porcine brucellosis, PB), the complex and dynamic nature of the epidemiology of animal brucellosis must be borne in mind when analyzing a given epidemiological setting since sometimes certain *Brucella* species can be found in host species other than their preferred ones (for example, *B. abortus* may be the etiological agent of brucellosis in sheep [1, 2]). A perfect example of this complex situation is shown by the increasing importance of *B. melitensis* in cattle in some Mediterranean countries, e.g., Saudi Arabia, Egypt, and Kuwait [3-5].

The need for a control of animal brucellosis has been a major concern since the first report describing the implication of animals in the epidemiology of the disease in 1905 [6]. Although brucellosis had been known for centuries as a chronic recurrent febrile disease mostly present in the Mediterranean region, it was not until the end of the 19th century when it was recognized as a dramatic disease wreaking havoc among the British army stationed in Malta. In 1887, Sir David Bruce (a British surgeon whose surname would later give name to the genus) isolated the etiological agent (firstly named as *Micrococcus melitensis*) of the infectious disease that was affecting an increasing number of soldiers in the island [7]. In 1904, the great concern raised by the impact of brucellosis in Malta contributed to the constitution of the "Mediterranean Fever Commission (MFC)," with Sir Bruce as the president of the organization. One of the main aims of the commission was to identify the sources of infection of the disease, an objective that was finally achieved by serendipity [8]: Sir Themistocles Zammit, a Maltese doctor member of MFC, included goats for experiments due to the temporary shortage of monkeys, traditionally used for in vivo studies. Surprisingly, agglutinins and bacteria were detected in the blood and milk of infected goats, thus suggesting these small ruminants were susceptible to the disease and a potential source of infection [6]. The ban on the consumption of Maltese goat milk among British soldiers was the first step for the control of this dramatic zoonosis in the island [9], one of the first preventive measures to control the transmission of *Brucella* from animals to humans.

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Brucellosis can be considered a paradigm of the need for a "One World, One Health" strategy given that the only approach to achieve the control and subsequent eradication of this zoonotic disease is the cooperation between the industry, producers, and public and animal health authorities [10]. Human-to-human transmission, although possible and occasionally reported due to transplantation, sexual contact, and lactation [11-14], has an insignificant impact on the epidemiology of the disease since humans are traditionally considered dead-end hosts [15]. The major sources of infection for human are therefore infected animals, not only due to direct contact but also - and most importantly - through the consumption of raw dairy products [3]. Traditionally, the main etiological agent of human brucellosis is *B. melitensis*, although a relevant role of *B. abortus* and *B. suis* (mainly biovars 1 and 3) has also been described. Although approximately 500,000 new human cases of brucellosis are reported every year around the world [16, 17], underdiagnosis/underreporting of human brucellosis is a major issue in many regions [18, 19]. The control of the disease in humans is impaired by the lack of available vaccines [20], thus leaving the control of animal brucellosis as the most effective strategy to prevent human infection [19, 21, 22]. In fact, surveillance systems for human brucellosis can act as sentinel tools of the situation of the disease in animals since the occurrence of human cases can be one of the first indicators of the presence of disease in the animal population [23], and likewise, a decreasing trend in the number of human cases may suggest that brucellosis control campaigns are effective [24, 25].

However, and despite its crucial importance from the public health perspective, the justification for the control of animal brucellosis rests not only on its zoonotic nature but also in the severe losses that its presence entails. Economic costs derived from the presence of *Brucella* infection in animals are derived from the direct consequences of the disease (abortion, infertility, reduction of milk production, orchitis, epididymitis, etc.) and the indirect losses (replacement of reactors, costs associated to control/eradications programs, movement restrictions, trade limitations, etc. [23, 26]). In addition, brucellosis has been recognized as a neglected zoonotic disease that contributes to the perpetuation of the poverty in endemic regions of low-income countries, compromising their economic development [27, 28]. Moreover, costs due to human brucellosis, as the investments on treatments, prevention of the disease, and loss of productivity are other overheads attributable to animal brucellosis.

2. Control and eradication strategies for animal brucellosis

Despite the huge efforts invested on the control of animal brucellosis, results have not always matched the expectations, particularly in the case of ovine and caprine brucellosis, in which control has proven to be more challenging than that of bovine brucellosis due to *B. abortus*. This situation may be the consequence of the combined effect of several factors, including those inherent to the disease regardless of the etiological agent/infected host [existence of a prolonged latent period often associated with lack of serological responses [29] and limited sensitivity of some diagnostic tests in certain epidemiological situations [30, 31] and also other factors associated with the etiological agent (environmental resistance of *B. melitensis* and *B.*

abortus [32]) and the host (traditional farming practices as communal pastures and transhumance practices, typical of small ruminants [33, 34]).

Still, three major strategies have been demonstrated as effective tools to control brucellosis in domestic animals when used in combination:

1. Strict biosecurity at the farm level
2. Test and slaughter programs
3. Immunization of the susceptible population

The sole implementation of one of these measures is however much less effective since optimal results are obtained when at least two of them are applied jointly. Still, the best strategy will depend on the epidemiological situation in a given setting, the availability of resources, etc. [26]. Moreover, in addition to these "classical" strategies, other complementary tools should be considered to ensure the success of the program (animal identification, animal movement control, economic compensations, etc.) [33].

The present chapter will review the tools currently available to achieve the control and eradication of brucellosis in livestock (bovine, porcine, and small ruminant brucellosis), focusing on the advantages and drawbacks of the diagnostic tools and the immunization strategies the two main pillars in which control programs are based. New approaches based on the advance in the knowledge of different aspects of the disease and of their etiological agents will also be reviewed. Finally, the factors that should be considered when selecting the most suitable strategy for control of small ruminant, bovine, and porcine brucellosis and that often determine the success of the control/eradication efforts will be discussed.

2.1. Management and biosecurity

Management and hygienic measures against *Brucella* infection must be focused in diminishing the possibility of contact with viable *Brucella*, including both infected animals and contaminated environments.

The most frequent routes of entry of *Brucella* in a free farm are the following:

- Purchase of infected animals that can shed the bacteria to the environment, therefore exposing susceptible individuals. In ruminants, up to 10^{10}-10^{13} CFU/g of tissue and membranes of aborted fetus can be excreted during the clinical phase of the disease [35]. In swine, infected boar may excrete 10^{4}-10^{5} CFU/ml of semen, thus turning venereal transmission one of the most important routes of infection for *B. suis*, particularly in brucellosis-free settings in which artificial insemination can constitute an important risk factor [32].
- Contact with infected material, pastures, etc., due to the high environmental resistance of *Brucella* spp., which leads to its persistence outside the host for long periods, allowing a variety of transmission routes of *Brucella* (conjunctival, oral, and respiratory).

Thus, the use of appropriate biosecurity measures is of critical importance to prevent the entrance of the disease in a naïve epidemiological unit. These strategies include the imple-

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mentation of quarantine before the introduction of new animals, the separation of animals with an unknown/uncertain status, the control of animal movements, the adequate management of replacement, the isolation of pregnant females before parturition (particularly primiparous animals), and the strict quality/sanitary control of semen. In case of artificial insemination, avoid or limit the contact between livestock and wildlife in environments where wild animals have been seen to be a source of infection [23, 29, 36, 37].

In infected settings, in addition to the biosecurity recommendations cited above, hygienic measures are essential to limit and control the bacterial load in the environment, decreasing the possibility of contact with viable *Brucella* spp., and should be systematically implemented. Removal of abortion products, full cleaning and disinfection of premises, elimination of infected manure, and incineration of infected material are some examples of measures to attain this objective.

Certain management/farming practices (traditionally used in small flocks from endemic region of low-income countries), such as nomadism, mixing animals from different origins at grazing, and use of shared pastures, may favor transmission of the bacteria, thus hindering the effectiveness of control strategies [38, 39].

2.2. Test and slaughter programs

The main aim of this approach is the early detection and removal of possible sources of infection (infectious animals), thus avoiding circulation of *Brucella*. Despite the effectiveness of the diagnostic strategy used, there is always a certain risk of having infected animals that may remain as silent carriers [40] maintaining the pathogen in the flock and, if there is a drop in the immunity of the herd, may lead to an abortion storm. This strategy is most useful in low-prevalence settings where economic resources and veterinary expertise are available for its support [41]. Test and slaughter strategies may also be useful for the management of outbreaks, particularly when numbers of animals make the implementation of stamping-out measures unfeasible [42]. Although the tests used for the detection of infection can be classified according to different criteria, this section is organized based on its ability to detect the pathogen (direct tests) or the immune response induced in the infected host (indirect tests that can be further subdivided on account of the immune response they target, humoral or cellular). In some cases, the only measure that achieves complete elimination of the bacteria on the flock is the stamping out followed by a thorough cleaning and disinfection and replacement with *Brucella*-free animals [43].

2.2.1. Indirect diagnostic tests

Most of these techniques (especially those using inactivated whole-cell suspensions of *Brucella* as antigens) were developed for the diagnosis of bovine brucellosis in the first place and were further adapted later for its application in small ruminants and swine considering that the principle of all techniques is the same regardless of the *Brucella* species/host: all the major tests are based on the detection of antibodies against the smooth lipopolysaccharide (S-LPS; the immunodominant antigen of smooth *Brucella* species: *B. abortus*, *B. melitensis*, and *B. suis*) [44].

The outer-polysaccharide chain (O-PS), the main antigen moiety of smooth *Brucella* LPS, shows a different proportion of α-1,2 and α-1,3 linkages dividing strains in two groups: A-strains or M-strains, depending on the quantitative distribution of A and M antigens in the smooth species. Thus, the suitability of *Brucella* antigens recovered from M-dominant strains (such as *B. abortus* biovar 1, typically used for rose bengal test (RBT), complement fixation test (CFT), and some ELISA techniques) for the diagnosis of brucellosis due A-dominant strains (e.g., *B. melitensis* biovar 1) has been questioned [45]. In fact, some authors have shown that RB and CFT may present an impaired diagnostic performance when used on small ruminants (particularly in goats) compared with that observed in cattle [46]. However, other studies have demonstrated that the use of *B. abortus* antigens for performing RB and other serological techniques allows an appropriate sensitivity for its use in *B. melitensis* diagnosis in small ruminants [47, 48]. A possible explanation for this finding would be the existence of common epitopes (C-antigen) present in the LPS of A- and M-dominant *Brucella* strains [49].

The accuracy of the diagnostic tests in the control/eradication programs of brucellosis is an essential component in the success of test and control strategies [50]. Although the diagnostic performance of most of the currently available diagnostic techniques has been demonstrated to be adequate, there are some epidemiological situations in which serological diagnosis may have some limitations. For example, silent carriers of the disease (e.g., infants infected congenitally *in utero* or by ingestion of contaminated colostrum/milk) may remain seronegative until a reproductive failure occurs [51]. In addition, positive results may not always be indicative of an active infection [52] since the occurrence of diagnostic interferences associated with exposure to other Gram-negative bacteria has been extensively demonstrated [53]. These microorganisms have LPS molecules similar to the *Brucella*-LPS in the outer membrane and may induce the production of cross-reacting antibodies, thus leading to false-positive results in the traditional serological techniques for brucellosis diagnosis. Different bacteria (including *Escherichia coli* O:116, *E. coli* O:157, and *Vibrio cholerae* O:1) have been recognized as a potential cause of this diagnostic interference, but *Yersinia enterocolitica* serotype O:9 is considered the main agent compromising the diagnostic specificity of serological tests [54]. This is a major challenge in the case of swine brucellosis [32, 55], especially when biovar 2 occurs, because its LPS antigenic structure is more similar to *Y. enterocolitica* than to other biovars of *B. suis* [56]. Most of these cross-reacting antibodies belong to the M isotype, and therefore the implementation of serological techniques based on the predominant detection of IgG1 is potentially more useful [53].

The diagnostic specificity of serological tests may also be compromised by the occurrence of false-positive reactions caused by antibodies induced by vaccine strains (mainly the smooth *B. melitensis* Rev. 1 and *B. abortus* S19, the most widely used strains for immunization against small ruminants and bovine brucellosis, respectively).

There is not a single serological test that can detect 100% of the infected population, and even used in combination may miss up to 30% of the infected animals using some serological tests [45], although its sensitivity at the herd level is much higher. In fact, in the frame of control and eradication programs, serological results are often interpreted at the herd level [46]; hence, the presence of one reactor involves the possible exposure to *Brucella* spp. of all animals of the

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flock that are therefore considered suspected and put under restriction measures until the infection is considered cleared in the herd/flock.

However, limitations in terms of the specificity of serological tests (such as those stated before) lead to the need of confirming the infection using other tools (epidemiological evidences of exposures and, preferably, isolation of the bacteria) at the herd level. Culture is considered the gold standard technique for the confirmation of the infection due to its high specificity [57], although this method is not free of drawbacks (see below).

The existence of international standards for diagnostic tests, vaccines requirements, reporting data systems, etc., is a cornerstone in the implementation of adequate control/eradication programs for animal brucellosis, making possible the harmonization of animal health systems worldwide [58]. For animal brucellosis, the OIE is the main standard-setting body publishing.

Factors including cost, time between sampling and achievement of test results, resource requirements, and ease of performance are other aspects that need to be considered in the choice of the best serological methods for the diagnosis of animal brucellosis in a given setting [50].

2.2.1.1. Indirect diagnostic tests based of humoral immune response against Brucella spp.

Rose bengal test (RBT)

RBT is a slide agglutination technique based on the use of cells of *B. abortus* S99 or S1119-3 stained with 1% rose bengal as the antigen [59]. It mainly detects IgM and IgG1 [30] mostly produced against LPS from smooth *Brucella* [60]. The antigen is buffered at an acidic pH (3.65 ± 0.5) in order to limit the agglutination due to IgM increasing the specificity of the technique [53]. The main advantage of this test is its high sensitivity, which makes it very suitable as a first screening test for determining the presence/absence of infection at the herd level [59, 61, 62]. The sensitivity (Se) and specificity (Sp) of this test have been extensively evaluated in different epidemiological scenarios in cattle (with Se estimates ranging from 53% to 100% and Sp from 79% to 100% [63-70]), small ruminants [Se ranges between 75.8% and 100% and Sp between 68.4% and 100% [66, 67, 71-73]), and pigs (Se estimates from 66% to 100% and Sp from 45.5% to 100% [32, 55, 74-76]). Its few technical requirements, speed for obtaining results, and low cost coupled with its performance have made this diagnostic tool one of the most widely used tests for the diagnosis of animal brucellosis. Its main disadvantages are related to its limited specificity in certain conditions, often due to cross-reacting antibodies derived from previous vaccination or exposure to other Gram-negative bacteria [77]. In many countries, the RBT is used as a first screening tool so that positive reactions are subsequently confirmed with an additional technique applied in series if no evidences of infection exist in the epidemiological unit. Due to its reliability, the RBT is one of the techniques described for international trade in cattle, small ruminants, and swine [59, 62].

Complement Fixation Test (CFT)

This test is based on the ability of the complement (obtained from guinea pig serum) to lyse erythrocytes (traditionally sheep erythrocytes sensitized with hemolysin) in the absence of an antibody-antigen complex. When *Brucella*-specific antibodies are present in the serum being analyzed, they bind to the *Brucella* antigens (whole cells) provided externally, forming antigen-antibody complexes that then bind to the complement [78]. In this case, the amount of complement in the reaction decreases, preventing its attachment to the hemolysin and the subsequent lysis of the erythrocytes that are added in the final stage. Procedures for performing this technique are described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [59, 61, 62]. Many studies have evaluated the sensitivity and specificity of this technique, showing a slightly to moderately better overall performance in general compared with the RBT (Se estimates vary from 80.6% to 98.79% in small ruminants [66, 67, 79] and from 53% to 100% in cattle [63, 68, 69], and Sp values range between 65.5% and 100% in small ruminants [66, 67, 79] and between 80% and 100% in cattle [63, 68, 69]). Due to its high performance, the CFT has been broadly used as a confirmatory technique in the programs of eradication and control of brucellosis in cattle and small ruminants (for confirmation of RBT-positive results) and is prescribed for international trade. However, under field conditions, the CFT may show a lower sensitivity than RBT, which makes it a very reliable test at the herd level but more limited at the individual level [35, 80]. The sensitivity of this test is also directly related to the stage of infection of the animal being tested, with higher values for animals in an acute phase than in chronically infected ruminants [81]. The disadvantages of this technique are derived from the subjectivity of its interpretation (especially for low titers), the complexity of its performance, and the unavoidable variability of reagents, procedures, etc., that makes comparison of results difficult [82]. Moreover, false positives may also occur in animals sensitized with *Y. enterocolitica* O:9 [83] and in those immunized with smooth vaccines (Rev. 1 in small ruminants or S19 in cattle [59, 80]) as in the case of RBT. The natural anticomplementary activity of sheep serum must also be considered because it can lead to the occurrence of false-positive reactions [23]. Furthermore, the swine complement interacts with the guinea pig complement used in the test, resulting in a procomplementary activity that may reduce its sensitivity in pigs [32, 84-86]. Nevertheless, the CFT is still contemplated by the OIE as a diagnostic technique of election for swine [61], with a wide range of specificity estimates (from 40% to 100% depending on the study [32, 87, 88]).

Enzyme-Linked Immunosorbent Assay (ELISA)

Since the first ELISA-based technique in the diagnosis of brucellosis [89], many studies have assessed its usefulness. ELISAs based on the use of LPS as the main antigen are considered the most useful and are widely employed. This has led to the commercialization of many ELISA kits (including indirect and competition ELISAs) for its use in domestic species. Based on currently available information, the sensitivity of ELISA-based techniques is similar or greater than that of RB and FC tests, although the actual values may vary depending on the study (from 67% to 100% in small ruminants [71, 72, 90, 91], from 67.9% to 100% in cattle [63, 65, 68, 69], and from 68.5% to 100% in pigs [32, 55, 74, 75]). The specificity of these tests is also considered very high (from 94.5% to 100% in small ruminants [71, 73, 91, 92], from 90.5% to 100% in cattle [63, 65, 68, 69], and from 76% to 100% in pigs [32, 55, 74, 75]). For these reasons,

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ELISAs are considered by the OIE adequate tests for the diagnosis of bovine, small ruminants, and porcine brucellosis [59, 61, 62], with the advantages of the possible automation of the technique, its reproducibility, and its objective interpretation (since a quantitative value is obtained as the final result). However, ELISAs based on LPS may suffer from the same diagnostic interference problems due to vaccine antibodies [53] and false-positive serological reactors (FPRS) associated with the presence of antibodies against *Y. enterocolitica* O:9 or other Gram-negative bacteria (mainly in swine [55] and cattle [70]) as those described for RBT and FCT. Due to these diagnostic limitations, the use of other antigens (e.g., proteins of outer or cytoplasmic membrane) has been evaluated for the development of new ELISA techniques for brucellosis diagnosis. However, in general, the serological response induced by these non-LPS antigens is considered to be heterogeneous and delayed compared with that observed using LPS antigen, which may affect the performance of diagnostic tests in the context of a control and eradication program. Still, extensive research has been carried out in this regard, with numerous studies focusing on the development of ELISAs based on proteins that could help to differentiate vaccinated from infected cattle, sheep, and goats (see section Development of Diagnostic Techniques for the Differentiation of Infected and Vaccinated Animals (DIVA)). Other studies have focused on protein targets that may allow the differentiation of *Brucella*-infected animals from those exposed to other bacteria, usually *Y. enterocolitica* O:9, mainly in pigs and cattle (BP26 protein, cytoplasmic protein extracts). However, these tests have generally shown a lower sensitivity than the ELISAs based on the LPS [70, 76].

Other indirect diagnostic tests based on the humoral immune response against *Brucella* spp.

Besides the abovementioned serological techniques, other tests have been developed for the diagnosis of *Brucella* spp. in animals. Among those, one of the most frequently used techniques is the fluorescence polarization assay (FPA), considered by the OIE as a valid technique for international trade in cattle, small ruminants, and pigs [59, 61, 62]. This test has similar or superior diagnostic performance compared with conventional techniques (CFT/RBT), being technically easier to perform [50, 75, 79, 93, 94]. Thus, in bovine, its use is recommended instead the CFT [59]. Another technique that has been widely used in certain countries is the standard agglutination test (SAT). This assay shows, however, a lower sensitivity and specificity than others, such as RBT and FCT in small ruminants [46, 95-97]. Still, its usefulness in these animal species has been demonstrated [98]. In cattle, SAT has been widely employed in brucellosis eradication programs as a confirmatory test [63, 99]. However, the OIE does not recommend nowadays the use of SAT for international trade in cattle [59] due to its lower specificity compared to CFT [59]. Nevertheless, SAT (and also the FPA) has demonstrated a higher sensitivity compared with the CFT for the detection of recently infected animals [100]. In swine, SAT has also been widely used [101, 102], and it is currently referred as a confirmatory technique in the EU international trade in pigs, although the OIE highlights that it may show a limited specificity due to the presence of nonspecific IgM antibodies in swine serum [61]. Finally, the milk ring test (MRT) has demonstrated its usefulness to detect specific antibodies against *Brucella* spp. in bovine milk samples used at herd level [59, 103], although its sensitivity may be impaired when it is used in large herds, and its use in small flocks is not recommended due to the expected low specificity [59, 62].

2.2.1.2. Indirect diagnostic tests based on the cellular immune response against Brucella spp.

Brucellin Skin Test (BST)

This diagnostic test involves the intradermal injection of a mixture of cytosolic proteins generally extracted from a *B. melitensis* rough strain (e.g., *B. melitensis* B115-Brucellin INRA) [104] that will induce a delayed hypersensitivity reaction (Type IV) [35] if the animal has previously contacted the bacteria. Recent studies have studied the usefulness of *B. abortus ΔmanBcore* as an alternative source of antigens for the BST for swine brucellosis diagnosis [87, 105]. The inoculation is usually performed in the lower eyelid in small ruminants [62], in the caudal fold, in the skin of the flank or the side of the neck in cattle [59], and in the base of the ear or the side of the tail in pigs [61]. Free-LPS antigens are required to prevent the subsequent induction of cross-reacting antibodies that may interfere with the traditional serological tests [81]. The main characteristic of BST is its high specificity, which makes it a useful tool to elucidate problems due to FPSR caused by *Y. enterocolitica* O:9 [106, 107], especially in brucellosis-free areas [25, 44], and as a complementary technique to serological tests [108]. However, occasional false-positive reactions due to *Ochrobactrum anthropi* infection have also been described [109]. In addition, its use is exclusively indicated in unvaccinated animals. This technique may be of particular interest to detect animals in the early and chronic stages of infection [23, 108], but its limited sensitivity makes its interpretation at the individual level difficult. Still, it is considered a suitable tool for *Brucella* diagnosis at herd level. Herd history, clinical signs, and serological or bacteriological results should be considered in the interpretation of the BST results. In ruminants, BST is recognized as an alternative technique for international trade [62]. In the case of swine brucellosis, it is not currently regarded as an official test, although its usefulness is supported by the EFSA and the OIE as a complementary diagnostic test [32, 61].

Interferon Gamma (IFN-γ) Detection

The interferon gamma is one of the most important cytokines involved in the cellular immune response against *Brucella* [110-112]. The usefulness of the in vitro quantification of IFN-γ produced by cells from naturally infected animals for monitoring the cellular immune response against this pathogen has been studied in cattle [113], porcine [107], and sheep [114, 115]. The levels of IFN-γ measured in samples stimulated with a *Brucella*-specific antigen (and that should be especially high in cases of previous contact with *Brucella* spp.) are typically quantified using an ELISA. This technique has been suggested as a complementary test to the serological techniques routinely used for the diagnosis of brucellosis in ruminants in the case of false-positive reactors due to its specificity [113], although there is still some controversy about its usefulness as a routine diagnostic technique for brucellosis.

2.2.2. Direct diagnostic tests

These techniques are based on the direct detection of the etiologic agent or its genetic material in clinical samples from infected animals. *Brucella* isolation is the gold standard for confirmation of infection [59, 61, 62, 116], and its use is recommended to improve the efficiency of eradication plans [25]. The use of specific staining methods (e.g., Stamp's method) in clinical specimens may reveal the existence of *Brucella* in clinical samples, although the limited

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sensitivity and specificity (especially due to the potential presence of other abortifacient pathogens such as *Chlamydia abortus* and *Coxiella burnetii* [62] that may lead to false-positive results) of this diagnostic approach can compromise its usefulness for the routine laboratory diagnosis of brucellosis.

2.2.2.1. Bacteriology

The World Health Organization classifies the *Brucella* genus bacteria as a risk agent III [62], and therefore high laboratory and training requirements are needed to handle the pathogen and potentially contaminated samples. The preferred samples for the direct detection of *Brucella* spp. are as follows: (i) in live animals: vaginal swab, milk, placenta, and fetus aborted samples (especially lung, spleen, and stomach contents) and (ii) postmortem: the reticuloendothelial system, udder, uterus, testis, and epididymis [25, 59, 61, 62, 117]. The possible contamination of clinical samples requires the use of selective media, frequently Thayer-Martin and Farrel media, for the isolation of *Brucella* spp. [59, 61, 62]. However, the presence of some components in the Farrel medium may have an inhibitory effect on some *B. abortus* and *B. melitensis* strains as well as in the *B. suis* biovar 2 [118]. Thus, the combination of more than one selective culture media and inoculation of at least two plates of each sample, including also a nonselective media for the isolation of *B. suis* biovar 2, may help to increase the chance of isolation of the pathogen [119]. The addition of serum or blood (usually from horse) to the media promotes the growth of *Brucella* spp. [82] and is necessary in the case of *B. abortus* biovar 2 [59]. Moreover, some strains of *B. abortus* (biovars 1-4 and 9) need a 5-10% CO_2 atmosphere [59]. Microbiological culture has a limited sensitivity in certain epidemiological situations, such as chronically infected animals that have typically low bacterial load in their samples. For this reason, negative culture result should not be considered as definitive evidence to rule out *Brucella* infection in an animal/herd [29, 120]. In fact, the sensitivity of culture is highly variable, depending on the stage of infection, the specimens analyzed, and the number of samples cultured [100, 102, 118, 121-123].

2.2.2.2. Molecular detection

DNA-based techniques allow the detection of nonviable *Brucella* or highly contaminated samples that may be challenging if handled using a traditionally culture-based diagnostic approach. In addition, molecular techniques may be applied at large scale through the use of automated equipment. PCR techniques are also routinely used for identification of *Brucella*-compatible isolates cultured from clinical samples. A considerable number of molecular techniques based on the PCR amplification of *Brucella* spp. DNA has been described, although often these techniques have not been fully validated on field samples as direct detection tests, hampering their implementation as routine techniques for *Brucella* detection [124]. Despite the high homology of DNA among *Brucella* species, different PCR protocols to identify different species/biovar and even vaccine/field strains have been described [125-133]. The efficiency in the DNA extraction procedure (which depends on the nature of the sample [134]) determines substantially the results of the PCR assays [135-137].

2.3. Immunization

Since Eduard Jenner described the first vaccine against smallpox in 1796, the immunization against infectious agents has become a cornerstone in the control of many of the most important infectious diseases. As general rule, vaccination efforts are often focused in the most susceptible individuals in order to stimulate a protective immune response against the pathogen of interest. However, in the case of animal brucellosis, vaccination campaigns target a less susceptible population (nulliparous nonpregnant young sheep, goats, and cows), while vaccination of the most susceptible subset is avoided due to the side effects of vaccination of pregnant animals (abortion, bacteria excretion, environmental contamination, etc.) [26].

The characteristics of the "ideal vaccine against brucellosis" include the following: (i) to induce a solid and long-lasting protection against the infection by different *Brucella* species without the need of re-vaccinations, (ii) to be innocuous regardless of the reproductive stage of the animal (so that there is no induction of abortion in pregnant animals, and mass vaccination can be applied if needed), (iii) to have no or very residual virulence for human and be susceptible to the antibiotics typically used to treat human brucellosis, (iv) to avoid the induction of cross-reacting antibodies in the serological techniques traditionally used in control/eradication programs, (v) to be affordable, and (vi) to possess stability at different environmental temperatures [138, 139]. Unfortunately, this ideal vaccine is far from those currently available for the control of animal brucellosis. For example, in the case of small ruminant brucellosis, Rev. 1 has been recognized as the most effective vaccine currently available considering its efficacy to prevent the abortion and transmission of *B. melitensis*, but it cannot be applied in pregnant females and is pathogenic for humans, among other nondesirable side effects [139]. The smooth S19 strain is the most widely employed vaccine in the case of bovine brucellosis, and even though it is currently considered the reference strain, it presents similar limitations as the Rev. 1 despite its demonstrated efficacy. For these reasons, the rough strain RB51 is increasingly used in some regions of the world as an alternative for vaccination against bovine brucellosis since its use does not induce the production of cross-reacting antibodies, even though its efficacy is still under discussion in certain epidemiological situations [140]. In general, the inability to vaccinate pregnant animals is a major disadvantage in animal brucellosis vaccination since it complicates achieving a quick increase in the proportion of the resistant subset of the population, which could lead to an average benefit-cost ratio of 3.2 (2.27-4.37) by reducing (52%) the transmission between animals by means of mass vaccination [21].

The success of the use of live vaccines for immunization against animal brucellosis is based on a balance between an adequate colonization of the host, triggering a solid protection against infection with other *Brucella* field strains, and a limited replication that minimizes the residual virulence of these vaccine strains [141]. Although immunological mechanisms induced by living vaccine have not been completely elucidated, live vaccines should stimulate the innate immunity, activate CD8+ and CD4+ cells, and generate an adequate population of memory cells, among other mechanisms, to induce a solid protection [139].

Most of the drawbacks associated with the use of live vaccines could be overcome with the use of killed bacteria or subunit vaccines; however, the ability of these inactivated vaccines to

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provide a solid and long-lasting immune response against *Brucella* has been traditionally considered lower than that triggered by live attenuated vaccines. New approaches are being investigated to elude the main drawbacks of inactivated vaccines to induce protective immunity in domestic livestock.

In the following section, the main drawbacks of currently available vaccines (Rev. 1 for SRB and S19/RB51 for BB) are described, as well as some of the new approaches followed to solve some of these shortcomings.

2.3.1. Residual virulence of attenuated live vaccines

The pathogenicity of Rev. 1 and S19, evident in its ability to induce abortion, has been a limitation since they were first used. However, in the case of Rev. 1, some authors have reported significant differences in its residual virulence. In a study performed to assess the immunogenicity and residual virulence of Rev. 1 strains recovered from different geographic origins, important differences between strains were identified [142], which may explain, at least in part, the diversity of results sometimes reported using this strain. For this reason, according to the OIE recommendations, strict quality controls must be maintained during the Rev. 1 production process in order to confirm that all batches have the typical characteristic of the original *B. melitensis* biovar 1 Rev. 1 strain. Residual virulence of Rev. 1 and S19 is reflected in the following sections.

2.3.1.1. Abortifacient effect

The ability of Rev. 1 to induce abortions was reported in the first studies, around the middle of the last century [143]. This adverse effect is especially significant when females are immunized around midpregnancy [144]. The abortifacient effect of the S19 strain has been demonstrated, although it is considered reduced and lower than that observed after Rev. 1 vaccination in general [140]. The appearance of a small percentage of vaccinated animals that may remain persistently infected with the S19 strain has also been described and may lead to abortions in adulthood [145]. In addition, vaccination may involve the excretion of the vaccine strain in milk and vaginal secretions [146, 147]. Even so, although the possible excretion of Rev. 1 during the lactation has been demonstrated, there is some disagreement about the relevance of this phenomenon [148].

Different strategies have been explored in order to avoid the abortifacient effect of Rev. 1 and S19, as described in the following sections.

Restriction of vaccination to replacement females (nulliparous, nonpregnant)

The protection induced by vaccination at full doses in 3-6-month-old animals using Rev. 1 [149, 150] and in 3-8-month-old animals using S19 [59, 151] is sufficient to induce a long-lasting protection. However, this control strategy may be problematic in certain epidemiological situations (e.g., high prevalence of brucellosis when mass vaccination is the only strategy to control the disease [24]).

Application of reduced doses of vaccine

Different studies have demonstrated the suitability of the immunization with reduced doses as an alternative to full dosages of vaccines, assuming that the afforded immunity was adequate and/or the abortion rate associated with the vaccination was significantly lower [59, 152-154]. However, field results obtained after the application of Rev. 1/S19 at reduced doses has led to a divergence of opinions among different experts about its usefulness and implications: many authors have reported a significant number of reproductive failures/vaccine excretion [155-157], advising against the use of this strategy in brucellosis control programs. In addition, some authors have showed that this immunization strategy using Rev. 1/S19 induces an inadequate protection against *B. melitensis*/*B. abortus* infection [156-159]. Additionally, reduced doses may not avoid the induction of persistent antibody titters when Rev. 1 and S19 are applied in adult animals, leading to a diagnostic interference problem with traditional serological techniques [59, 153, 156]. However, other experts have argued in favor of the efficacy of this approach as part of some brucellosis control programs [160-163]. In the case of S19, some experiments have shown that the best protection was obtained by subcutaneous vaccination of calves at full doses followed by conjunctival administration of a booster reduced dose [164].

Modification of route of vaccination

Although the application of Rev. 1 by the conjunctival route has been demonstrated to reduce the number of reproductive failures induced after subcutaneously immunization of pregnant females, the safety of this immunization strategy is not enough to be used regardless of the physiological stage of females [144, 146]. In bovine, S19 conjunctival vaccination does not avoid completely abortions in pregnant females [155], although it is considered that the conjunctival administration in reduced doses (5×10^9 microorganisms) is an alternative route in adult vaccination due to the reduction of abortion rates [59].

2.3.1.2. Other side effects

In addition to the induction of reproductive failures, other adverse effects, as transient periods of fever and anorexia or swelling at the vaccination site, have been reported after Rev. 1 vaccination [31]. Some studies have showed a risk of environmental contamination with the vaccine strain after Rev. 1 vaccination of young females, suggesting these animals are shedding the Rev. 1 strain and could therefore be a source of infection for other susceptible individuals [165, 166]. However, other authors have reported that conjunctival vaccination of nonpregnant animals immunized is safe for the environment [167, 168]. Some occasional contradictory effects (as orchitis) in billy goat and ram have also been reported [169, 170]. Regarding to the S19 vaccine, other side effects reported include the appearance of arthropathies associated with type III hypersensitivity reactions [171], persistent orchitis in males [153], and significant reduction in milk production and udder infections [140, 172].

2.3.2. Zoonotic potential and antibiotic resistance of attenuated living vaccines

A limited number of human brucellosis due to Rev. 1 [173, 174] and S19 [175-177] infection has been reported. The risk of infection for human and environmental contamination is neverthe-

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less reduced if the adequate biosafety practices during handling these live vaccines are followed. Rev. 1 and RB51 carry antibiotic resistance genes to streptomycin and rifampicin, respectively, which are used in the treatment of human brucellosis. In the case of S19, resistance to penicillin G (associated with an increased virulence in mice) has been evidenced [178].

2.3.3. Instability of vaccine strains

The possible instability of Rev. 1 is due to its tendency to dissociate into a rough phenotype, reducing its effectiveness in the field [138]. Several strategies such as the addition of 5% of serum (from horse, cattle, or rabbit) on the solid medium used for Rev. 1 production have been demonstrated to be useful to prevent this dissociation [179]. Biological quality and efficacy of S19 may be affected by inadequate subculture or maintenance conditions [180]. In the field, the main strategy to prevent this instability is the strict control of refrigeration temperatures for storage during all the process until the moment of inoculation (what could be problematic in certain circumstances, leading to the application of low-quality vaccines) [181]. In addition, preliminary observations also suggest that a reversion to a more pathogenic phenotype of the Rev. 1 strain is also possible [166], although the genetic stability and homogeneity of Rev. 1 strains is considered demonstrated [182].

2.3.4. Diagnostic interference in serological techniques

The smooth LPS antibodies induced after Rev. 1 and S19 vaccination are indistinguishable from those triggered after infection with a smooth field *Brucella* strain. The Rev. 1-derived antibodies may be detected even 4 years after vaccination depending on the age of the animal at vaccination, the immunization doses, and the serological technique being used [183]. For this reason, vaccination using smooth Rev. 1 and S19 creates a diagnostic interference problem when test-and-slaughter (T&S) programs based on the use of traditional serological techniques are in place, complicating the combination of these two strategies. The following sections present three possible approaches to overcome this diagnostic interference problem.

2.3.4.1. Reduction of the serological response induced by Rev. 1 vaccination

- Application of reduced doses of vaccine (see section Application of Reduced Doses of Vaccine)
- Restriction of vaccination to replacement females [nulliparous, nonpregnant; see section ´Restriction of Vaccination to Replacement Females (Nulliparous, Nonpregnant)].
- Modification of the route of vaccination

The subcutaneous route, traditionally used for the immunization with Rev. 1 and S19 in small ruminants and cattle, respectively, triggers a solid and long-lasting serological response (at least up to 20 months after vaccination in goats [184] and 22 months in cattle [185]). However, in the last 30 years, the usefulness of the alternative use of the conjunctival route has been demonstrated in cattle [164, 186] and small ruminants [187]. Vaccination of young animals using Rev. 1 inoculated by the conjunctival route induces a limited serological response but is

able to trigger an adequate protection for at least the two first pregnancies following vaccination) [188]. The serological response induced by conjunctival Rev. 1 vaccination is more long-lasting in adults, therefore leading to the potential occurrence of diagnostic interference problems [187], although these would be still more limited than those observed after its application by the subcutaneous route [189].

In the case of the S19 vaccination, the OIE suggests an alternative immunization protocol based on the application of 5×10^9 CFUs conjunctively in bovines regardless of the age of the animal [59]. Thus, as mentioned above, the vaccination protocol based on subcutaneous application of S19 during calfhood and conjunctival revaccination using reduced doses in adult animals has been proved as a suitable strategy for the control of BB in endemic populations, which avoids the need for slaughter of false-positive reactors [164]

2.3.4.2. Development of diagnostic techniques for the Differentiation of Infected and Vaccinated Animals (DIVA)

Different studies have been carried out to identify immunogenic non-LPS components that could allow the development of diagnostic techniques able to detect the serological response induced by *Brucella* field strains only. Some studies have showed promising results, but most of the authors also report limitations (such as less intense and more heterogeneous antibody response, lack of information about diagnostic performance under field conditions, etc.) that make their routine implementation on animal brucellosis control/eradication campaigns difficult. In the case of S19, the use of competition ELISAs coated with S-LPS allows the removal of most false-positive responses due to vaccination-derived cross-reacting antibodies [59, 93, 190, 191].

The use of several non-LPS *Brucella* components as the main antigen for DIVA serological techniques has been described, including the following:

- *Proteins.* The detection of immunogenic proteins involved in the humoral immune response of the host in the outer membrane of *Brucella* was the first step in the development of alternative serological methods for brucellosis diagnosis. In the case of *B. melitensis,* CP28 (also called BP26) was recognized as a good candidate to distinguish between Rev. 1 vaccinated and infected animals [192]. Several indirect [193, 194] and competitive [195] ELISAs have been developed to assess the suitability of CP28 as a diagnostic target for small ruminant brucellosis diagnosis, showing a lower diagnostic performance than that detected using ELISAs based on the LPS antibodies response [196]. In the case of *B. abortus,* different protein antigens (*N*-formylperosamine *O*-polysaccharide-protein conjugate [197], an extract of cytoplasmic proteins of *Brucella* and an 18-kDa cytoplasmic protein [198]) have been described as suitable candidates for the development of new immunological tests for screening and infection confirmatory diagnosis. Additionally, Pajuaba *et al.* showed that the AHRPO protein (protein A-horseradish peroxidase) is an adequate conjugate for the development of an indirect ELISA (that uses *B. abortus* S-LPS as an antigen) to differentiate S19 vaccinated and *B. abortus*-infected animals due to a preferential detection of the IgG2 isotype, a valuable marker of *Brucella* infection [199].

- *Polysaccharides.* The native hapten, one of the polysaccharides present in the surface of smooth *Brucella* strains, has also been used as a potential antigen in an agar gel immunodiffusion (AGID) test to solve the potential diagnostic interference problem. When antibodies against this polysaccharide are present (samples from *B. abortus*/*B. melitensis*-infected ruminants), a ring of precipitations appears in the agar [200, 201]. If samples from vaccinated (and uninfected) animals are analyzed several months after vaccination, no antibodies against the polysaccharide would be detected. In adult cattle, subcutaneous vaccination with reduced doses does not produce positive reactions (except for those animals that are infected and excrete the bacteria in milk [202]). The AGID test has demonstrated its usefulness to solve diagnostic interference problems in bovine and small ruminants [77, 203, 204], although some authors have also reported a sensitivity too limited for its wide-scale application [53]. This technique has also been demonstrated to be suitable to elucidate cases of FPSR due to *Y. enterocolitica* infection in cattle [70].

2.3.4.3. Use of rough vaccine strains (lack of O-PS or O-PS defective strains)

Rough *Brucella* strains are naturally devoid of the O-PS, which confers them a more granular and dull surface compared with those carrying complete S-LPS (smooth strains). Due to the lack of antigenic O-PS, these rough mutants may not induce anti-O-PS antibodies and, therefore, do not cause diagnostic interferences in most serodiagnostic tests. The rough phenotype can be observed by crystal violet staining (rough strains uptake the staining turning to red/violet) or autoagglutination in acriflavine solution [140]. In 1997, a WHO Consultation on the Development of New/Improved Brucellosis Vaccines encouraged the need of studies on live attenuated rough vaccines as an alternative to smooth strains for immunization against brucellosis [205]. Mutant rough *Brucella* strains are obtained by (i) natural dissociation of smooth (S) to rough (R) phenotype and subsequent repeated in vivo or in vitro passages of R mutants or (ii) genetic modification of the sequence of genes involved in the synthesis/transportation of component(s) of the smooth LPS [140]. Despite the potential advantages associated with the use of rough strains as vaccine candidates, some potential undesirable traits may make their application in the field difficult:

- The attenuation of R mutants has been associated to modifications in the outer membrane of *Brucella* and, therefore, possible changes in their interaction with components of the immune system of the host [206]. If the attenuation is too high, the rapid clearance of the R mutants in the host may lead to an insufficient protection [140]. However, natural rough *Brucella* species (*B. canis* and *B. ovis*) are virulent for their preferred hosts, and in fact, the role of LPS in the pathogenesis of *Brucella* is controversial: although the protection against *Brucella* is mainly mediated by the cellular immune response (triggered primarily by bacterial antigenic proteins), the humoral response (antibodies) may also have a role in the resistance against *Brucella*, as demonstrated by passive immunity experiment with sera against the LPS [207-209] and even with antibodies against the rough *B. melitensis* B115 strain [210] (see section Control of Small Ruminant Brucellosis).

- The diagnostic interference associated to the smooth phenotype of *Brucella* may not be totally avoided with the use of rough strains since animals vaccinated with R strains have showed occasional reactions to the S-LPS ELISA [211].

- The possible zoonotic potential of rough *Brucella* strains may limit their large-scale use. In the case of human brucellosis due to rough *Brucella* strains, traditional serological techniques, based on the detection of antibodies against S-LPS, may not detect the infection leading to misdiagnosis [140]. A possible alternative in this case would be the use of specific techniques for the detection of antibody response against rough *Brucella*, such as the CFT developed for the detection of rough B115 *B. melitensis* infection in sheep [212].

In the case of bovine brucellosis, the suitability of several rough vaccines (45/20, *pgm* mutant, RB51, *Brucella abortus* strain 82) for the control of the infection due to *B. abortus* has been evaluated [213-216]. However, RB51 vaccine could be considered the main representative example of the potential usefulness of rough *Brucella* vaccines in the battle against animal brucellosis. RB51 (R: rough; B: *Brucella*; 51: identification number of the laboratory of origin) is a stable rough rifampicin resistant *B. abortus* strain produced after repeated passages of *B. abortus* strain 2308 on trypticase soy supplemented with 1.5% agar and varying concentrations of rifampin or penicillin [215]. No O-PS antibodies were detected in rabbits, goats, and cattle after immunization with this strain [215, 217, 218], although some authors have reported low level of M-like O-chain in vaccinated species [219] and certain apparent anamnestic responses have been reported [220]. The induction of none to very limited vaccine-mediated abortions has been reported in cattle [221, 222], although contradictory evidences have also been reported [223]. In cattle, RB51 triggered an adequate protection against infection with virulent *B. abortus* strains [151, 224, 225], but certain concerns have been raised regarding its suitability in certain epidemiological situations in the field (see section Control of Bovine Brucellosis). RB51 has also been evaluated as a tool for the control of small ruminant and swine brucellosis. In the case of *B. suis*, some authors have demonstrated its usefulness preventing abortion in swine [226], although recent studies have demonstrated the inefficacy of parental RB51 vaccination to induce humoral or cell-mediated immune responses or to protect against abortion in a virulent challenge with *B suis* in domestic pigs [85]. Protection induced by RB51 vaccination against *B. melitensis* was also insufficient to consider it as a potential candidate in the prevention of ovine brucellosis [227], although the results regarding the suitability of this vaccine strain for the immunization of goats are controversial [140, 228, 229].

Many attempts have been conducted to achieve a stable immunogenic rough vaccine against *B. melitensis* using genetic engineering [VTRM1- *rfbU* mutant, *rpoB* mutant, *wa***, and *wzm* mutant, B115) [206, 211, 230-232]. In the case of swine brucellosis, a recent study of Stoffreger *et al*. has demonstrated that a rough *B. suis* strain (353-1), isolated from urine of a feral boar and prepared by propagation from the original isolation on Tryptose agar containing 5% bovine serum (TSA) at 37°C and 5% CO_2, can induce a significant immune response and confer a partial level of protection from a challenge with a virulent *B. suis* [233]. However, no rough vaccine candidate is currently recognized as a suitable alternative for immunization of sheep/goats and swine in the framework of an animal brucellosis control program.

3. Control of bovine brucellosis

The OIE has established the following requirements for a country/zone to be considered as free from this disease: (i) BB is declared notifiable; (ii) an official veterinary control is estab-

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lished in the entire bovine population, in which flock prevalence of disease is lower than 0.2%; (iii) no vaccination has been performed in the last 3 years (at least); (iv) all herds are subjected to periodical serological testing; (v) all reactors are culled; and (vi) new animals introduced in the region belong to officially brucellosis-free (OBF) herds (or free of brucellosis with vaccination). Some countries (France, Germany, Norway, Sweden, The Netherlands, Japan, Canada, Australia, and New Zealand, among others [59]) are considered to be OBF, but the disease is still present in many others despite the implementation of control/eradication programs. In general, measures against BB are based on vaccination (when the prevalence is high, in the initial steps of the control strategies) and test-and-slaughter programs (or herd depopulation when the disease prevalence is already very low and economic and technical resources are available) in the final stages of the eradication process prior to the achievement of the OBF status. These measures (vaccination, test and slaughter programs, and herd depopulation) alone cannot however be fully effective in eradicating the disease without the additional implementation of other complementary prevention measures, such as control of animal movements, use of surveillance systems, adequate laboratory support, etc.

Even though the role of wildlife reservoirs in the epidemiology of bovine brucellosis is considered minimal in most countries in the world, several wild species in certain scenarios can act as reservoirs of the disease for cattle, such as wild bison elk in the Greater Yellowstone Area (USA) [234]. In these situations, wild populations should be considered in the design of BB control programs.

In the case of BB, two vaccines are available for the control of *B. abortus* in cattle: the S19 (smooth) and the RB51 (rough) strains, which have demonstrated their effectiveness in the reduction of the number of abortion, transmission, excretion, etc., in certain epidemiological situations [235].

The S19 strain has been the main vaccine used against bovine brucellosis in many countries for more than 50 years. Numerous studies have demonstrated the usefulness of S19 calfhood vaccination with full doses (10^{10} CFUs) to protect them against *B. abortus* infection during their whole productive lifespan [236]. However, as mentioned before, its smooth nature may lead to the induction of O-PS antibodies that may persist until the adulthood, causing a diagnostic interference problem. Nevertheless, adult vaccination may be occasionally recommended in certain scenarios (high prevalence settings in which a rapid impact on disease spread is needed, large herds in which test-and-slaughter strategies are not feasible). Still, adult vaccination remains an emergency measure since the induction of vaccine antibodies and the possible abortifacient effect of S19 in pregnant cows are two very important side effects that would be associated with it. The use of reduced doses of S19 vaccine (10^{9} CFUs) in adults can partially limit those side effects, but its full usefulness is debatable [214, 237]. Immunization with RB51 vaccine (rough strain with a minimal expression of O-PS) for the control of *B. abortus* infection has emerged as an alternative to S19/stamping out in certain scenarios [238]. However, its true usefulness and its ability to induce a degree of protection equivalent to that induced with the S19 vaccine are still under discussion. The safety and protection afforded by RB51 against infection with *B. abortus* have been demonstrated in experimental conditions [224, 239, 240], but some of these results are still considered controversial [140]. Field evidences suggest RB51

could be considered a useful complementary tool for BB control [151, 238, 241], but the impossibility of comparing the progress achieved by strategies with and without RB51 vaccination under the exact same conditions in most cases impairs the evaluation of the relative contribution of RB51 vaccination to the overall success of an eradication program [242]. Recent studies have showed a potential beneficial effect of an RB51 booster vaccination in adult cattle after S19 calfhood vaccination to control *B. abortus* infection [243].

Even though *B. abortus* is undoubtedly the main concern when dealing with infection in cattle, *B. melitensis* is also a potential etiologic agent of brucellosis in bovine. The control of *B. melitensis* infection in cattle is hampered by the lack of information on important aspects of its epidemiology [244]. Outbreaks due to *B. melitensis* in cattle are often attributed to the presence of infected small ruminants in the surrounding area [245], suggesting that the key for the control of this pathogen in bovine will be the control of the disease in ovine and caprine flocks, as for human brucellosis.

4. Control of small ruminant brucellosis

Even though the most important factors that have to be considered for the control of SRB have been well characterized, socioeconomic factors have influenced the choice of the most suitable control measures in most of the endemic areas. For example, the existence of pastoral ecosystems, a traditional management practice in low-income areas where *B. melitensis* is endemic, has contributed to the perpetuation of the disease due to the difficulty of detecting the disease in the early stages of infection and of implementing control measures [246]. In addition, management practices favoring the mixing of animals with different origins (for example, the existence of communal grazing pastures) modify the traditional concept of minimal epidemiological unit of intervention (an essential key to consider by decision makers of animal brucellosis control programs). The minimal epidemiological unit is defined as "any number of animals that are held, kept or handled in such a manner that they share the same risk of exposure to brucellosis" [247] and can therefore include the flock or supraflock levels.

Although extremely important, the prevalence of brucellosis is not the only issue to consider for decision makers: the organization of the veterinary services, the availability of a suitable animal identification system and of the economic and technical resources that these measures require, the involvement of producers, veterinarians, and administrative authorities, etc., are all key aspects that must be evaluated [24]. When the prevalence of brucellosis is high and/or the socioeconomic resources are limited, the vaccination is the most suitable tool for the control of the disease. Despite its drawbacks, Rev. 1 is the best currently available vaccine to immunize sheep and goats against *B. melitensis*. In order to minimize the diagnostic interference problem due to Rev. 1 cross-reacting antibodies, a restricted vaccination strategy has been implemented in most of the regions where test and slaughter policy is applied as part of the SRB control program. The vaccine is administered by the conjunctival route in young (<6 months) female animals at doses of 0.5-2 × 10^9 CFU/animal (also controlling the impact of the abortifacient effect of Rev. 1). Some authors [24, 31] have suggested that restricted vaccination may be

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insufficient in certain epidemiological situations (such as high prevalence regions or where nomadism is practiced) and mass vaccination should be implemented instead. The immunization at full doses by the conjunctival route during the prebreeding period and late lambing season would be the most suitable approach for whole flock vaccination because it may prevent reproductive failures due to Rev. 1 vaccination [156]. Blasco *et al.* [24] have described two methods to carry the whole flock vaccination in the case of sheep and goats: (i) mass vaccination of males and females every two years avoiding lambing period (and considering 15-25% annual replacement) and (ii) restricted vaccination of replacement animals (at least 8-10 years) except the first year when all animals would be immunized regardless of their age. When the prevalence has decreased, a restricted Rev. 1 strategy could be then implemented. In general, a vaccination program against SRB should last at least 8-10 years in order to assure an adequate vaccine coverage [24]. The correct identification of vaccinated animals is an important factor to achieve this vaccination coverage that will grant an adequate immunity at the flock level [33].

Despite the success achieved thanks to Rev. 1 vaccination in many regions of the world, the drawbacks associated with its use have prompted the study of alternative vaccines against *B. melitensis*: DNA vaccines, subunits vaccines, outer membrane vesicles, smooth *B. melitensis* mutants (BP26, P39, Omp25, and *ΔpurE201*), nondividing but metabolically active gamma-irradiated *Brucella melitensis*, and attenuated live rough vaccines [230, 231, 248-254]. Among rough vaccines against SRB, the B115 strain has demonstrated to induce a solid protection not only against *B. melitensis* but also against *B. ovis* and *B. abortus* in the murine model. An abortifacient effect was demonstrated in sheep after subcutaneous vaccination with B115, although the interference diagnostic problem was drastically reduced [255]. Still, the possible occurrence of reversions of its phenotype (rough to smooth) in vivo could affect its attenuation, thus highlighting the need of further studies to better determine the stability of B115 in sheep before giving it further consideration as a possible candidate for SRB vaccination.

When certain epidemiological indicators (disease prevalence, number of cases of human brucellosis, etc.) suggest the control of brucellosis has been achieved, the next step is to success in the eradication of the disease [24]. A restricted Rev. 1 immunization strategy should be implemented exclusively when the prevalence is already low after 6-12 years of whole-flock vaccination, a strict control of animal movements and an accurate animal identification system exist, and veterinary services and economic resources are available. The change from mass vaccination to restricted vaccination is a critical step in the control/eradication programs since it may suppose the culling of a high number of false seropositive adult vaccinated animals, even if conjunctival vaccination is performed. Two different approaches have been proposed to minimize this shortcoming [24]: (i) lack of testing for two years after the change from mass to restricted vaccination (after which a strict test and slaughter policy would be implemented, where all CFT-positive animals are culled and their flocks of origin retested until 100% of animals are seronegative at least two consecutive tests) and (ii) the implementation of a serological test capable of differentiate vaccinated from infected small ruminants as part of the test-and-slaughter strategy as soon as the whole flock vaccination is stopped (such as the radial immunodiffusion tests whose usefulness has been discussed before). The restricted vaccination should be maintained after the achievement of a close to zero brucellosis prevalence. After

that, if the epidemiological situation is maintained and the risk of reintroduction of *B. melitensis* has been minimized, the prohibition of vaccination and the implementation of an eradication program based exclusively on test and slaughter policy could be proposed. Vaccination coverage, the implementation of other complementary measures, and the availability of an effective monitoring strategy should be considered by decision makers to establish the moment of vaccination ban [34]. The availability of economic and technical resources (economic compensation for farmers, strict animal movement control, etc.) is a cornerstone to ensure the success of a test-and-slaughter strategy in regions with close to zero disease prevalence.

5. Control of swine brucellosis

Porcine brucellosis is a notifiable OIE-listed disease [256]. However, in many countries, no specific control and eradication programs are implemented, and there is no obligation to conduct monitoring/surveillance strategies on the swine population for *Brucella* infection. Therefore, the actual prevalence of porcine brucellosis in many areas is not exactly known and probably is often underestimated. For example, in the case of EU Members States, the estimates of the prevalence of swine brucellosis vary depending on the region, but the current situation of *B. suis* in swine population is not totally recognized due to the lack of systematic epidemiologic data [32]. One of the main differences in the control and eradication of this pathology in swine compared to ruminant brucellosis is the lack of availability of safe and effective vaccines against *B. suis* as Rev. 1 for *B. melitensis* and S19/RB51 for *B. abortus* [32, 61]. Nowadays, the only country where vaccination against swine brucellosis is applied is China, where a national control program against this disease (mainly caused by *B. suis* biovar 1) was initiated due to the high rate of human brucellosis cases and the economic losses in animal production. In addition to animal vaccination, other strategies such as culling of all aborted females, separation and removal of infected animals, and quarantine policies were also implemented [257]. Since 1981, pigs have been immunized in China with a live attenuated *B. suis*S2 vaccine. In some brucellosis-infected areas with low prevalence, vaccination was only applied to young livestock so that test-and-slaughter programs could also be implemented. In other areas in which this was not feasible, an intermittent vaccination policy was applied, and the vaccinated animals were not tested to avoid the diagnostic interference problems caused by the use of this vaccine. The S2 vaccine can be administered by parenteral route or *per os*, although it should be applied at high doses (20×10^{10} FCU/animals) in two doses [257]. One of the side effects of this vaccine is that it can cause abortions in pregnant sows, and despite its wide use in China for decades, OIE does not recommend its use for the control of swine brucellosis, partly due to the absence of trials demonstrating its efficacy and safety in controlled conditions [61].

In general terms, although in most countries there are no specific plans for the control and eradication of porcine brucellosis, the most frequent approach for swine brucellosis control is the test and slaughter strategy, similar to that used in ruminants. Thus, eradication requires the identification of infected animals, the progressive elimination of reactors from the herd, and their replacement with noninfected animals (testing all animals and applying a quarantine

period) [32]. The application of whole-herd slaughter when reactors are detected should be considered as one strategy that would reduce the risk of circulation within the drove and its spread to other holdings due to undetected infected animals. However, this measure is often not feasible because of the economic implications for farmers. On the other hand, whole-herd depopulation and repopulation with noninfected animals must be implemented in countries considered free of the disease [258]. An example of a country where this measure is used as part of its control an eradication program is the USA, where *B. suis* has been eradicated from commercial pigs [259], thanks to the great efforts invested in the last decades. In 1972, the U.S. Department of Agriculture National Brucellosis Eradication Program implemented in ruminants was expanded to cover swine herds, based on the serological testing and removal of reactors. Depending on the epidemiological situation, three strategies were implemented [260]: (i) whole-herd depopulation of infected herds and repopulation, recommended for commercial herds and seed stock producers who wish to eliminate swine brucellosis from their population rapidly, and often the solution in the final efforts at eradication; (ii) exceptionally, in herds with only one or a few reactors (and no clinical signs of swine brucellosis), frequent test and removal of reactors, although this option was not generally recommended; and (iii) offspring segregation: this plan was recommended where valuable bloodlines had to be saved and weaned pigs (usually negative when tested at weaning) were allowed to be moved to separate, clean premises (animals had to be retested at least once prior to breeding to tested, clean boars).

As part of a control program, certain conditions and testing are required to classify a herd as swine brucellosis free. Thus, herds may be validated as swine brucellosis free by conducting a complete herd test with negative results. Validation may be maintained by periodical testing of the whole herd with negative results. Besides USA, some other countries in America (such as Cuba and Panama) [261], where porcine brucellosis is present, have also implemented control and eradication programs based on these same approaches. Some countries and regions offer the farmers the option of implementing voluntary programs covered by the authorities in order to certify the flocks as swine brucellosis-free herds. These voluntary programs are based on the same principles as those used in the eradication and control programs, consisting of periodic serological testing of all animals in the herd and removal of reactors. This measure has been implemented in Argentina, being mandatory for local genetic suppliers and breeding animals destined for sale, fairs, auctions, and exhibitions [262]. Another example of voluntary program is in Australia, accepted by all states and nowadays implemented in Queensland, where *B. suis* is an enzootic disease of feral pigs [263]. Farms included in the Australian voluntary program should buy breeding stock only from herds registered in this scheme. If infection is detected in the herd, then the accreditation is withdrawn until all reactors are removed and the herd tested back to accreditation standards [264]. Another fundamental key for the control of swine brucellosis is the control of artificial insemination centers because they may be an important source of infection to many animals [265]. Therefore, control measures implemented in these centers are especially restrictive and have specific regulations for each country or region. These measures are based on serological testing and quarantine of all introduced animals (that must come from accredited *B. suis*-free farms),

continuous serological monitoring of the whole population of the center, and preparation of semen doses following certain requirements [32].

Control of wildlife reservoirs is an important part of the swine brucellosis control and eradication in regions where the disease is widespread in wildlife and may get in contact with the commercial swine population. For example, in USA, where swine brucellosis remains endemic in feral swine [266], the control of PB in this wild species is included in the current program of control and eradication, with specific measures regarding the restriction of feral swine movement between states, including test and slaughter and control contact with domestic pigs. According to the OIE, treatment with antibiotics is not being implemented anywhere as a control measure [256]. Despite its potential application to control an outbreak at the farm level [267], antibiotic therapy is currently strongly discouraged because it does not allow the total clearance of the infection and involves the use of high doses with a considerable cost and poses some additional problems due to the limits of maximum residue in animals destined to human consumption.

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