



Comparative Review of Brucellosis in Small Domestic Ruminants

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Brucella melitensis and *Brucella ovis* are the primary etiological agents of brucellosis in small domestic ruminants. *B. melitensis* was first isolated in 1887 by David Bruce in Malta Island from spleens of four soldiers, while *B. ovis* was originally isolated in Australia and New Zealand in early 1950's from ovine abortion and rams epididymitis. Today, both agents are distributed worldwide: *B. melitensis* remains endemic and associated with an extensive negative impact on the productivity of flocks in some regions, and *B. ovis* is still present in most sheep-raising regions in the world. Despite being species of the same bacterial genus, *B. melitensis* and *B. ovis* have extensive differences in their cultural and biochemical characteristics (smooth vs. rough colonial phases, serum and CO₂ dependence for *in vitro* growth, carbohydrate metabolism), host preference (female goat and sheep vs. rams), the outcome of infection (abortion vs. epididymitis), and their zoonotic potential. Some of these differences can be explained at the bacterial genomic level, but the role of the host genome in promoting or preventing interaction with pathogens is largely unknown. Diagnostic techniques and measures to prevent and control brucellosis in small ruminants vary, with *B. melitensis* having more available tools for detection and prevention than *B. ovis*. This review summarizes and analyzes current available information on: (1) the similarities and differences between these two etiological agents of brucellosis in small ruminants, (2) the outcomes after their interaction with different preferred hosts and current diagnostic methodologies, (3) the prevention and control measures, and (4) alerting animal producers about the disease and raise awareness in the research community for future innovative activities.

Keywords: *Brucella melitensis*, *Brucella ovis*, genomics, goats, sheep, pathogenesis

INTRODUCTION

Brucellosis is a worldwide, chronic infectious disease caused by small aerobic, non-motile, Gram-negative coccobacilli of the genus *Brucella*. There are 12 established species within the genus that are recognized based on preferential host specificity (1). Goats and ewes are the preferred hosts for *Brucella melitensis* whereas rams are for *Brucella ovis*, although small domestic ruminants may be infected by other *Brucella* species.

B. melitensis infection causes abortion, stillbirths and the birth of weak offspring, and occasionally epididymo-orchitis in goats and sheep (2) and is the most virulent *Brucella* species for humans (zoonotic), responsible for a severely debilitating and disabling illness that results in high morbidity with low mortality (3). *B. melitensis* has been controlled

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in most industrialized countries; however, it remains endemic and associated with an extensive negative impact on the productivity of flocks in low and middle-income nations, where goats and sheep are the major livestock species and the main economical livelihood, such as the Mediterranean region, the Middle East, Central Asia, Sub-Saharan Africa, and parts of Latin America (4).

On the contrary, *B. ovis* seems to be non-pathogenic for humans and the main clinical sign of infection is epididymitis in rams, with occasional abortions in ewes and increased perinatal death (5) (**Figure 1**). In small ruminants, there are reports of *B. ovis*-specific antibodies in goats from Brazil (6) and Bulgaria (7), and evidence of seroprevalence in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) in the USA (8). However, the etiological agent has only been isolated in naturally-infected domestic sheep and farmed red deer (*Cervus elaphus*) (9). Experimental infection of male goats with *B. ovis* via a natural route of entry showed that bucks may become infected and shed the pathogen in semen, but the infection is transient and their role in the epidemiology of the disease is negligible (10, 11). *B. ovis* was first isolated and identified from ovine abortion and epididymitis in rams in Australia and New Zealand in early 1950's (12, 13). Following these first reports, *B. ovis* has been found worldwide distributed. Today, the disease probably occurs in most sheep-raising regions in the world, being currently present in, e.g., Australia, New Zealand, Russia, France, Spain, Portugal, South Africa, United States, Mexico, Argentina and Brazil (14).

In addition, small domestic ruminants may also be infected by other *Brucella* species. Goats and sheep may be susceptible to *B. abortus* infection under particular epidemiological situations (for instance, when they live in close contact with *B. abortus*-infected cattle or camelids) (15–17); however, these flocks will not sustain the infection in the absence of infected primary host (18). *B. suis* isolates from goats and sheep have been seldom reported (19–22), and to the best of our knowledge, no other *Brucella* species have been isolated from small domestic ruminants. Considering that small ruminants may act as occasional hosts for other species of the genus *Brucella*, this review will focus on brucellosis caused by *B. melitensis* and *B. ovis*. Throughout this review, we will present: (1) the differences and similarities of the etiological agents at the biochemical and genomic level, (2) the pathogenesis and clinical consequences in their primary hosts, (3) the indicative prevention and control measures for the disease, and (4) summary and analysis of current guidelines and awareness tools to alert animal producers about the disease and promote future innovative actions in the research community.

CULTURAL AND BIOCHEMICAL DIFFERENCES BETWEEN *B. MELITENSIS* AND *B. OVIS*

Bacterial isolation remains the gold standard for diagnosis of brucellosis. Isolation of *Brucella* is typically achieved from fresh milk samples and vaginal discharges of sheep and goats (2), while samples from lymph nodes (LNs), spleen, reproductive tract and udder are collected for culture at necropsy (23). On the other

hand, semen is the optimal sample for *Brucella* isolation in rams and goat bucks, whereas inguinal LNs, spleen, seminal vesicles and the epididymis are the preferred necropsy samples (24). The abomasal content and lungs, followed by the liver and spleen, are the preferred samples for *B. melitensis* and *B. ovis* isolation from an aborted fetus (2).

B. melitensis grows in non-enriched basal media such as *Brucella* medium base, tryptose -or tripticase- soy agar (TSA), blood agar base or Columbia agar, without serum or CO₂ enrichment (25). Contrarily, optimal growth of *B. ovis* occurs when culture media is enriched with 5–10% blood or serum (sheep > bovine or horse > fetal calf serum) and incubated at 37°C in a microaerophilic atmosphere (i.e., 10–20% CO₂). This CO₂-dependence of *B. ovis* is caused by a guanine insertion in BOV_RS08635 locus that expresses a carbonic anhydrase II defective enzyme (26). Unlike *B. melitensis*, *B. ovis* cannot grow on glucose or galactose as primary carbon source, and is defective in metabolisms of ribose and erythritol (27). Selective media containing antibiotics are effective in suppressing contamination, and therefore useful for the isolation of field specimens. In spite of being the most widely used selective media for primary *Brucella* isolation, Farrell's medium contains antibiotics in its composition that may inhibit the development of *B. ovis* and some *B. melitensis* strains (28). On the contrary, other selective media such as modified Thayer-Martin (mTM) or Skirrow agar facilitate *B. ovis* and *B. melitensis* isolation, although the control of overgrowth of contaminating microorganisms is less stringent (29). A more sensitive selective medium (CITA medium) for primary *Brucella* isolation from field veterinary samples, was more recently developed (30). This new culture medium, in comparison to mTM has a 7-fold higher concentration (mg/L) of a Gram-positive bactericidal antibiotic vancomycin, and the addition of antifungal agent amphotericin B. A presumptive fast bacteriological diagnosis can be made on smears from placentas, aborted fetuses or vaginal swabs stained with Stamp's modification of the Ziehl-Neelsen's technique, although this method is not specific and the results must be confirmed by culture or PCR (25).

On solid media, 3–4-day old *B. melitensis* colonies are convex, translucent, smooth, glistening, and circular, 0.5 to 1 mm in diameter, while *B. ovis* colonies are round, more opaque with a dry, yellowish-white granular (rough) appearance and up to 2 mm in diameter (25). This difference is because *B. melitensis* expresses a full lipopolysaccharide molecule (smooth, S-LPS) that is anchored in the outer membrane (OM), while *B. ovis* expresses LPS that lacks the O-antigen (rough, R-LPS). However, bacterial culture identification has a low sensitivity, is time consuming, and requires skilled technical personnel to safely handle samples and live bacteria. Moreover, *B. melitensis* is associated with biosafety concerns due to the high risk of acquired laboratory infection and must be handled under BSL3 conditions, making diagnosis by bacterial isolation impractical in many situations. As opposed to *B. ovis* which does not require biosafety measures due to its lack of zoonotic potential, isolation and handling can be conducted at BSL2 facilities.

B. melitensis and *B. ovis* are identical at the microscopic level: both are small coco-bacillus with a size of 0.5–0.7

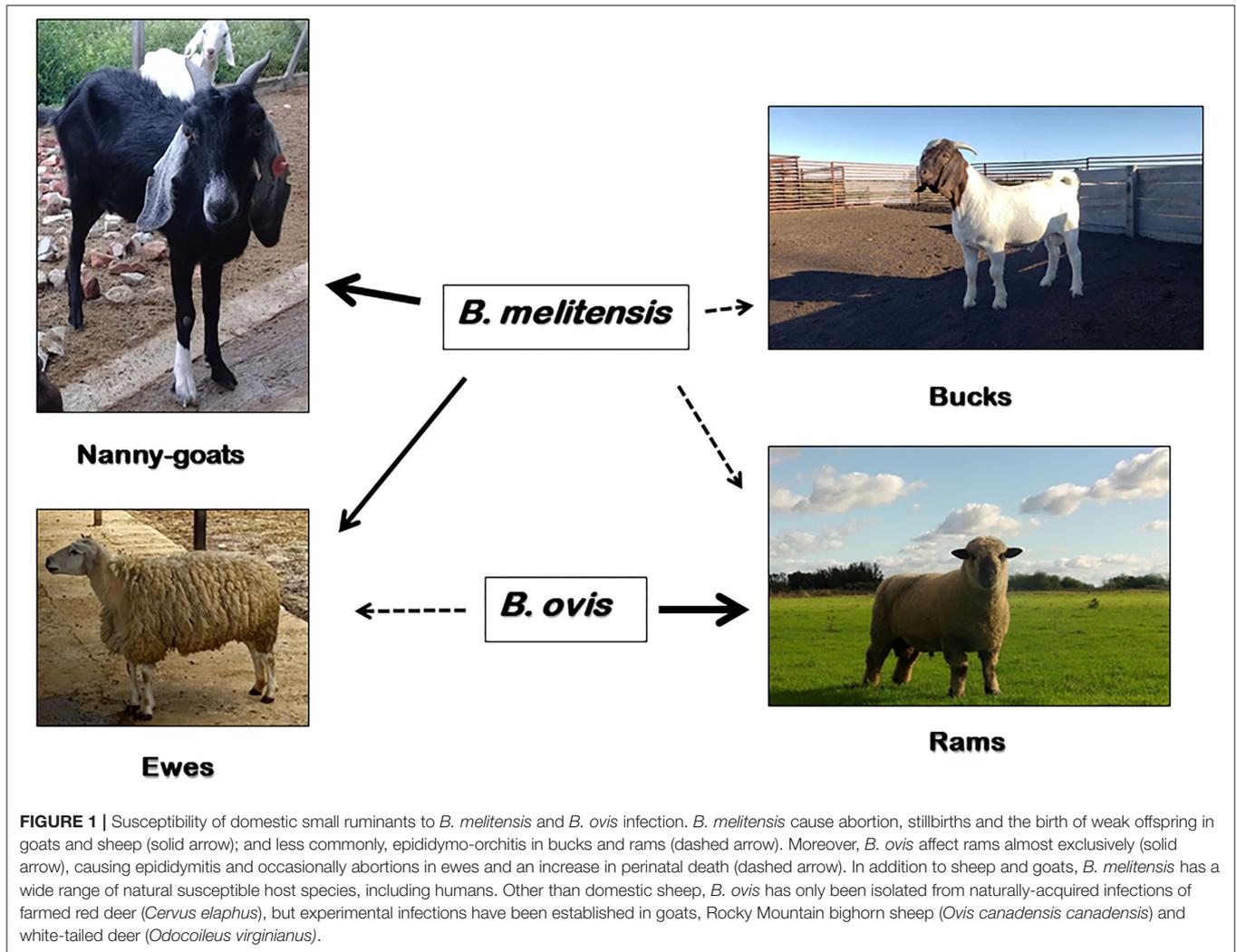


FIGURE 1 | Susceptibility of domestic small ruminants to *B. melitensis* and *B. ovis* infection. *B. melitensis* cause abortion, stillbirths and the birth of weak offspring in goats and sheep (solid arrow); and less commonly, epididymo-orchitis in bucks and rams (dashed arrow). Moreover, *B. ovis* affect rams almost exclusively (solid arrow), causing epididymitis and occasionally abortions in ewes and an increase in perinatal death (dashed arrow). In addition to sheep and goats, *B. melitensis* has a wide range of natural susceptible host species, including humans. Other than domestic sheep, *B. ovis* has only been isolated from naturally-acquired infections of farmed red deer (*Cervus elaphus*), but experimental infections have been established in goats, Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) and white-tailed deer (*Odocoileus virginianus*).

um in width and 0.6–1.5 um in length, non-capsulate, non-motile, and non-spore-forming, single or rarely forming short chains (25). For routine identification, only a few biochemical tests such as oxidase, urease, growth in presence of colorants and agglutination with monospecific serum are indicated to differentiate between both species. The main differences between these two *Brucella* species are summarized in **Table 1**.

PATHOGENESIS OF BRUCELLOSIS IN SMALL RUMINANTS

Susceptibility to *B. melitensis* infection increases from naïve sexually immature to sexually mature animals of either sex, and reaches its maximum in pregnant goat does and ewes. It is well-known that goats are more susceptible than sheep, and rams more resistant than ewes to the development of brucellosis disease caused by *B. melitensis*. A great variability in susceptibility to brucellosis was reported between sheep breeds, but not between breeds of goats. For example, Hampshire down and Texel breeds are more resistant to infection and consequently

less likely to abort, than some dairy breeds and Southwest Asia and Mediterranean fat-tailed breeds (3, 31).

The predominant route for *B. melitensis* infection under natural exposure is the alimentary tract (2). Animals, mainly goat does because of their naturally more curious character, are primarily infected by direct contact with aborted fetuses, placental membranes, vaginal discharges, or ingestion of contaminated water, pastures or colostrum. Once in the oral cavity, *B. melitensis* enters through the mucosal-associated lymphoid tissue (MALT) in the pharyngeal wall and colonizes, proliferates and persists for long periods of time in the lymph nodes (LNs) of the head (i.e., mandibular, parotid, and lateral and medial retropharyngeal LNs) (32). This MALT forms a ring around the pharyngeal wall called “Waldeyer’s ring,” that presents a similar histologic structure than the ileal Peyer’s patches, an anatomic structure easily penetrated by *B. melitensis* (33) (**Figures 2A–C**). Failure to eliminate *B. melitensis* at the primary line of defense results in bacterial escape through the efferent lymphatic vessels to the distal LNs or *via* blood to the systemic circulation (**Figure 2A**). *Brucella*

TABLE 1 | Main differences between *B. melitensis* and *B. ovis*.

Characteristics		<i>B. melitensis</i>	<i>B. ovis</i>
Host preference		Goats and sheep	Sheep (Ram)
Host susceptibility		Goats, sheep, cattle, human	Sheep, red deer
Zoonotic capacity		High	None
Biosecurity requirements		BSL 3	BSL 2
Biovars		3 (1–3)	1
Colony morphology		Smooth	Rough
CO ₂ dependence		–	+
Serum/blood requirement		–	+
Oxidase		+	–
Urease activity		+	–
Growth on	Basic fuchsin	+	Growth inhibited at ≥ 20 ug/ml (1/20.000)
	Methyl violet	+	–
Agglutination	Monospecific serum	Biovar 1: M Biovar 2: A Biovar 3: A–M	R (anti-rough)
	Acriflavine (1:1,000)	–	+

spreads free in plasma, inside erythrocytes or within phagocytic cells and can reach every organ, but persists in lymphoid tissues (LNs and spleen) and bone marrow (**Figure 2D**) and actively replicates in the pregnant uterus or, less frequently, in testis and epididymis. As strategy for persistence, *B. melitensis* prevents the apoptosis of infected mononuclear phagocytes to facilitate bacterial persistence in the reticuloendothelial system until the host becomes susceptible to placental colonization and replication during pregnancy (34). Considering that *Brucella* can reach 1×10^{10} colony-forming units (CFU)/ml in allantoic fluid and 1×10^{13} CFU/g of tissue in cotyledons (35, 36), and the infective dose of *B. melitensis* is 10 to 100 CFU (37), abortion and excretion of *B. melitensis* in vaginal discharges constitute an important new source of infection for other susceptible hosts.

Contrary to *B. melitensis*, *B. ovis* primarily infects males, and almost exclusively rams after puberty (5). *B. ovis* infection seems to be transient in bucks (10, 11), rarely cause abortions in ewes (38), and has apparently no clinical consequences in goat does. Similar to the *B. melitensis* infection, susceptibility to *B. ovis* infection may vary among sheep breed. In South Africa, the prevalence of clinical lesions was rated as Dorper > Karakul > Merino, and other authors also support that Merino breed is more resistant than other breeds of sheep (39–41), although potential flock confounding variables were not fully controlled.

Flocks become infected by the introduction of a *B. ovis*-infected ram, which disseminates the pathogen intermittently in semen, genital secretions and urine. Indirect venereal transmission between rams that have mated with the same ewes, or direct non-venereal transmission by homosexual behavior, or by the sniffing or licking of the preputial area of the infected ram are the most important ways of spreading the infection (5, 42). The pathogenesis of infection in rams has been poorly studied, but it appears

that after entering the body by penetrating the mucosae of the prepuce, rectum, oral or nasal cavity, *B. ovis* is phagocytosed by local antigen presenting cells, and transported to the regional lymph nodes. The possibility of infection *via* digestive route should not be considered, as lack of urease in *B. ovis* specie reduces its ability to survive in the acidic abomasal content (43). Progression of the disease is slow, and after 2–8 weeks of incubation period, the pathogen disseminates *via* lymphatic or blood vessels, free or in leukocytes, but in the long term only colonizes the genital organs and accessory sexual glands (seminal vesicles, bulbourethral—Cowper's—glands, ampullas of the *vas deferens*) (44). Colonization starts in the tail of epididymis and seminal vesicles, yet the molecular basis for targeting these tissues is unknown.

The pathogenesis of *B. ovis* infection in ewes has not been fully elucidated. Even though mating by infected rams or artificial insemination with infected sperm causes a low percentage of infection in ewes, contaminated semen may be the main source of infection of female sheep, as environmental contamination (water, pastures) does not seem to play a relevant role for *B. ovis* transmission (38, 45, 46), probably due to its deficient ability to infect *via* the oral route. After insemination with contaminated semen, *B. ovis* is transported and remain confined to the local drainage lymph nodes; later in pregnancy, bacteremia develops and *B. ovis* reaches the pregnant uterus and colonizes the fetus through chorion vessels. Opposite to what occurs with *B. melitensis* where subsequent reinvasion of the pregnant uterus and shedding may happen during following parturitions (2), *B. ovis* infection rarely extends from one pregnancy to the next (45, 47, 48). Therefore, the role of infected ewes in shedding and dissemination of the *B. ovis* is insignificant, as the ability of the pathogen to remain in the uterus or being excreted in vaginal discharges and milk seems to be limited, although exceptions were reported (49).

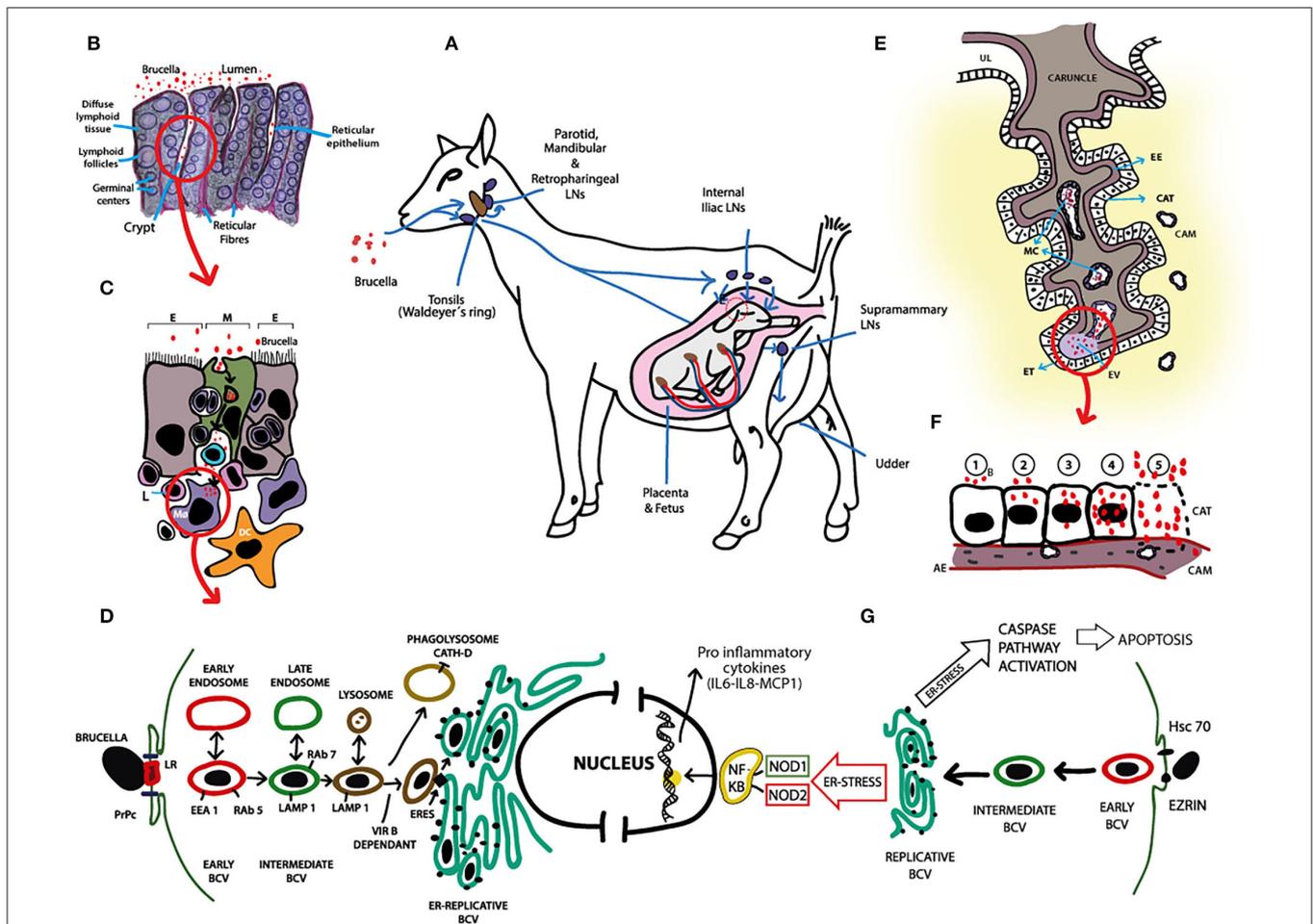


FIGURE 2 | Pathogenesis of *B. melitensis* infection in small ruminants. **(A)** *B. melitensis* infects small ruminants mainly via the alimentary tract. Once in the oral cavity, *B. melitensis* enters through the mucosa or the oro- and nasopharyngeal tonsils (Waldeyer's ring) and colonizes, proliferates and persists in the lymph nodes (LNs) of the head (i.e., mandibular, parotid, and lateral and medial retropharyngeal LNs). Failure to eliminate *B. melitensis* on this primary line of defense results in escape of the bacteria through the efferent lymphatic vessels to the distal LN or via blood to the general circulation. In pregnant females, *B. melitensis* colonizes placenta and induces abortion or stillbirth. *Brucella melitensis* also has affinity for lactating udder. Significant numbers of *B. melitensis* are excreted in vaginal discharges, aborted fetus, placenta and milk. **(B)** Illustration of histological structure of tonsils. *B. melitensis* is first seen in the lumen of the oral cavity. Subsequently, *B. melitensis* colonizes the crypts and invade through reticular epithelium. **(C)** Reticular epithelium is composed of scattered M cells (M; green), lymphoid cells and the epithelial cell types (E). M cells endocytose *B. melitensis* from the lumen, after which transcytosis and basolateral release occurs. Immediately, the agent is up taken by resident antigen presenting cells, i.e., macrophages (M ϕ) and dendritic cells (DC), which in turn activate the immune response in the underlying lymphoid follicles. M ϕ s and DCs traffic *B. melitensis* to other sites in the body or back through the reticular epithelium to the mucosal surface. **(D)** Schematic representation of *B. melitensis* intracellular trafficking in macrophages and evasion of killing. Following lipid rafts (LR)—and the prion protein (PrPc)—mediated internalization, *B. melitensis* is contained in an early vacuole (BCV: *Brucella* containing vacuole). This early vacuole sequentially interacts with cellular organelles (early and late endosomes, and lysosomes) and transiently acquires different markers (EEA1, Rab5, LAMP1, Rab7) in a *VirB* dependent-mechanism regulated by the pathogen effector proteins. To reach the replicative niche, BCV-LAMP1+ interacts with ER exit sites (ERES), and generates an ER-derived organelle permissive for *B. melitensis* replication (ER-Replicative BCV). Vacuoles containing *VirB*-deficient *B. melitensis* undergo phagolysosomal degradation. Spontaneous rough mutant generation might help parental pathogen release from infected M ϕ through lytic or non-lytic mechanisms, and the process repeats in other professional phagocytic cells. **(E)** Diagram of ruminant placentome with enlargement of one caruncular septa. *Brucella* invades placenta via maternal capillaries (MC). Extravasated (EV) maternal blood at the tip of the caruncular septa, along with *B. melitensis*, is phagocytosed by erythrophagocytic trophoblasts (ET). From these cells, *B. melitensis* spread infection to adjacent chorioallantoic trophoblasts (CAT). UL: Uterine lumen, EE: Endometrial epithelium, CAM: Chorioallantoic membrane. **(F)** Schematization of a complete cycle of *B. melitensis*-infected chorioallantoic trophoblast (CAT). 1: *B. melitensis* (B, red circles) adheres to the plasmatic membrane of CAT; 2: the bacteria are internalized and initial intracellular replication occurs; 3–4: Massive intracellular multiplication of the agent; 5: Apoptosis of CAT and release of huge number of *B. melitensis*. The cycle of endocytosis, intracellular replication and programmed cell death continues. CAM, Chorioallantoic membrane; AE, Allantoic epithelium. **(G)** Schematic representation of *B. melitensis* intracellular trafficking in chorioallantoic trophoblasts. Trophoblasts invasion of *B. melitensis* is mediated by heat shock cognate protein 70 (Hsc70) and Ezrin. From this point until reaching of the replicative niche, intracellular trafficking is similar than that reported in macrophages (i.e., BCV goes from early BCV -red- to intermediate BCV -green- and to replicative BCV). Intracellular presence of *Brucella* induces ER stress that triggers production of the pro-inflammatory cytokines in a nucleotide-binding oligomerization domain (NOD) 1/2—dependent manner via activation of NF- κ B pathway, and activates caspase pathway leading to chorioallantoic trophoblast apoptosis. These molecular responses of *B. melitensis*-infected trophoblasts may contribute to better understanding the pathogenesis of placentitis and abortion in small ruminant brucellosis.

CLINICAL CONSEQUENCES OF BRUCELLA-INFECTION IN DOMESTIC GOATS AND SHEEP

Abortion and Weak Offspring

Middle to late-term gestation abortion, stillbirths and the delivery of weak offspring sometimes followed by the retention of fetal membranes are the characteristic—and perhaps the only noticeable—clinical signs of the disease. However, these signs are not pathognomonic of brucellosis, and a differential diagnosis with other etiological agents is critical, although sometimes difficult to achieve in the clinical field. Therefore, it would be judicious to assume that all abortions are caused by a zoonotic agents and minimize exposure until a definitive diagnosis is made.

During bacteremia, *Brucella* enters the placenta through specialized trophoblastic cells located toward the fetal side, between the bases of cotyledonary villi of the placentomes that are involved in phagocytosis of macromolecules, especially extravasated maternal blood (Figure 2E). Thus, *Brucella* takes advantage of an important physiological mechanism for the trans-placental transport of iron needed by the developing fetus for erythropoiesis (50) to colonize naive targets. From these phagocytic trophoblasts, *Brucella* spreads and replicate into adjacent chorioallantoic trophoblasts (51). Massive intracellular multiplication induces apoptosis of trophoblasts due to endoplasmic reticulum stress (52) and release of huge numbers of microorganisms into the uterine lumen. Though, the cycles of endocytosis (or active penetration), intracellular replication and cell death continue (Figure 2F); consequently, placenta cotyledons heavily fill with brucellae and fetal invasion *via* bacteremia occur. Recent molecular studies demonstrated that *Brucella*-infected trophoblasts secrete proinflammatory chemokines such as IL6, IL8, GCP-2 and MCP-1, and hormones prolactin and estrogen, while the secretion of progesterone is inhibited (52–55) (Figure 2G). Altogether, these elements provide a local environment that contributes to abortion. Foci of apoptotic trophoblasts result in ulceration of the chorioallantoic membrane, which favors the hematogenous dissemination of *B. melitensis* to chorionic villi and fetal tissues (56, 57). Large numbers of brucellae in chorionic connective tissue gradually produce vasculitis and separation of fetal trophoblasts from maternal syncytial epithelium. Other histologic changes are associated with neutrophilic and histiocytic inflammatory infiltrate, and fibrin deposition, ranging from subacute to massive chronic purulent necrotizing cotyledonary placentitis. Eventually, when the number of viable placentomes is inadequate to sustain maternal-fetal interaction, fetal death and consequent abortion occurs. Fewer affected placentomes often results in underweight—weak newborns followed by a high neonatal mortality rate.

The mechanism that makes the pregnant uterus attractive to brucellae, especially to *B. melitensis*, is still undefined. High levels of erythritol in the ruminant placenta were originally postulated to explain the tropism and the subsequent accumulation of *Brucella* in this organ. In favor of this, the promoting role of

erythritol on *B. melitensis* expression on virulence genes, such as the Type IV secretion system VirB and flagellar proteins was also more recently demonstrated (58). However, there is increasing evidence that *B. melitensis* infect placenta and produce abortion in hosts such as humans (59, 60), mice and guinea pigs (61, 62), where erythritol is not a major component of the placenta. A clue to this mystery might have been recently deciphered by Barbier et al., who assigned a crucial role to the host polyol pathway enzyme aldose reductase, which catalyzes the synthesis of several alternative carbon (C) sources (including erythritol) for *Brucella* availability (63). Contrarily, *B. ovis* is unable to catabolize erythritol and many other gluconeogenic substrates and use them as C sources (27, 43), which may explain why the pregnant uterus is not as attractive to this *Brucella* specie as it is for others, resulting in lower incidence of abortion in infected ewes.

Epididymitis

B. ovis affect the epididymis, tunica vaginalis and testes producing infertility in rams. Earlier events include quantitative and qualitative alterations of semen characteristics (sperm motility, concentration, morphology, reduced fertility) which progress to palpable lesions in the scrotum with no changes in libido. Palpable lesions of the epididymis are occasionally bilateral and may vary from a slight enlargement to large indurations, with the tail affected more often than the head or the body (5). The testes may suffer atrophy and the tunica vaginalis is often thickened and fibrous, with extensive adhesions. Yet, *B. ovis*-infected rams may not always develop clinical manifestations; indeed, subclinical disease where seminal vesicles is the most common affected organ, appears to be the rule (42, 64). According to previous studies, *B. ovis* reach the genital tract by haematogenous spread, extravasate and colonize the interstitium. Earliest histologic changes are seen in the tail of epididymis, consisting in perivascular edema and diffuse interstitial accumulation of lymphocytes and plasmatic cells (65). Further degeneration and lysis of the epithelium allow extravasation and interstitial accumulation of spermatozoa, which increases the inflammatory response with tubular occlusion, formation of sperm granulomas and chronic pyogranulomatous epididymitis (64). From the time that *B. ovis* reaches the genital organs, the microorganism is chronically shed in semen and genital secretions, free or in infected phagocytes.

More rarely, *B. melitensis*-infected bucks and rams might present unilateral or bilateral epididymo-orchitis and can shed *Brucella* in semen for a year (66–68). Even though the epidemiological importance under natural conditions of the venereal route is usually disregarded, *B. melitensis*-contaminated semen used in artificial insemination could be a potential source of infection (69).

Other Clinical Consequences

B. melitensis, as well as *B. ovis*, have marked affinity for the lactating udder and supramammary LNs, although clinical findings are limited to decreased milk yield and slight enlargement of the LNs (47, 70). *Brucella*-infected phagocytic

leukocytes migrate from the systemic circulation to the mammary alveoli. In the ductal and alveolar lumen, *Brucella* replicates within phagocytes and upon host cells degeneration, brucellae are subsequently released and ingested by a new round of phagocytes. Part of the infected phagocytes is excreted in milk, and part migrates to the interstitium and reaches the supramammary LNs via the lymphatic stream (71). Milk components, like fat and casein, may reduce the effectiveness of intraphagocytic brucellacidal mechanisms, possibly contributing to the chronic infection of the udder and its regional LNs. Excretion of *Brucella* into the milk can occur intermittently throughout lactation and may continue during the following pregnancies, reaching considerable numbers (i.e., colony forming units / ml of milk) immediately after delivery and in late lactation (47, 72). Thus, ingestion of contaminated colostrum and milk could be a more frequent route of vertical transmission in small ruminants than *in utero* infection, although lambs born to infected dams may also passively acquire neutralizing antibodies (38, 73, 74). Lambs and kids born from infected females may become latent carriers until sexual maturity; some may develop clinical disease while others remain silently infected (69).

The natural *B. melitensis* infection in non-pregnant small ruminant females is usually asymptomatic (23). Similarly, systemic signs are rare in adult ewes and rams infected by *B. ovis*. Rams with subclinical disease become carriers or shedders of *B. ovis*, and must be diagnosed by culture or serological routine tests and eliminated from the flock.

GENOMIC BASES EXPLAIN DIFFERENCES IN PATHOGENICITY AND HOST SPECIFICITY BETWEEN *B. MELITENSIS* AND *B. OVIS*

B. melitensis comprises 3 biovars (bvs 1–3), that have similar virulence for goats and sheep. Historically, *B. melitensis* bv 1 is predominant in Latin America, bv 2 in Middle East together with bv 3, which is also more common in European and Africa Mediterranean countries, Eurasia and China, while bvs 1 and 3 seem to be equally present in India (4). Currently, there is only one bv for *B. ovis*. Few comparative genetic studies including both species have been done (Table 2). The genomes of these two *Brucella* species are composed of two circular chromosomes of ~2.1 (ChrI) and 1.2 Mb (ChrII) in size with 3,200–3,300 ORFs. Blasting the genome of *B. melitensis* against that of *B. ovis* reveals a high level of identity at the nucleotide level and almost an 80% of the annotated proteome is shared between them (43). This suggests that the differences in host preference and pathogenesis may be due to a relatively small number of genetic changes and differential mechanisms of gene regulation. Among the differences and probably related with the loss of virulence for most mammalian hosts, it is worth mentioning that *B. ovis* ATCC25840 genome has 264 unique—and 539 missing—annotated protein coding genes as compared to *B. melitensis* 16M genome (43). A comparative whole-genome

hybridization study reported that *B. ovis* REO198 genome lacks 80 ORFs distributed in five genomic islands (GI-1, –2, –5, –7, and –9) relative to *B. melitensis* 16M genome (1, 75). Among them, the loss of GI-2 and –5 were proposed as critical for its non-zoonotic nature. GI-2 contains two ORFs that encode for glycosyltransferase enzymes involved in LPS biosynthesis (BME_RS04960 –*wboB*- and BME_RS04965 –*wboA*-) and other ORF encoding for a porin family protein implicated in host cell interaction (BME_RS05010 –Omp25b-). In addition, GI-5 carries homologs of the ABC-type transporters such as Dpp, Opp, and Pot systems, important for attachment to host cell and intracellular survival in other bacteria (1, 75, 76). Contrarily, a highly conserved 26.5 kb GI is present in chromosome II of *B. ovis* strains (*B. ovis* pathogenicity island 1; BOPI-1), but not in *B. melitensis*. This GI contains genes that encoded for an ABC transporter (abcEDCBA), an antitoxin of the toxin-antitoxin system Phd/YefM family (BOV_RS12860), and several pseudogenes and hypothetical proteins (43).

Similar to other host-specific pathogens whose genomes often show signs of genomic decay related to their divergence from a generalist ancestor along with a change in their route of transmission (78), the more restricted tissue tropism (male genital tract) and narrow host range (rams) of *B. ovis* appears to be associated with its genome degradation (1). The higher number of pseudogenes (244 vs. 163) in *B. ovis* ATCC25840 genome with respect to *B. melitensis* 16M is directly related to the higher number of transposable elements (38 vs. 7) (43). This difference in the number of active insertion sequences favors genomic reduction and consequent loss of virulence (76). Some of the functional *B. melitensis* genes that are pseudogenes in *B. ovis* genome are involved in oral infection ability (*ureC1*), transport and metabolisms of carbohydrates as erythritol (*eryA*) and glucose (*gluP*), cell envelope structure (BME_RS14295, *omp31*) and cytochrome oxidase activity (*ccoO*). Moreover, contrary to *B. melitensis* 16M, *B. ovis* ATCC25840 genome also has four inactivated genes (*prlS*, *tceS*, *moaR*, and *stcA*) encoding for protein members of two components systems (TCS), signal transduction mechanisms with important virulence regulatory functions in *Brucella* spp. (77). Indeed, the inactivation of *tceS* and *prlS* may also play a role in the virulence restrictions exhibited by *B. ovis*, since these genes are necessary for the persistence of *B. melitensis* in mice (79, 80).

Despite differences in virulence among *B. melitensis* bvs. have not been reported to date, the absence of a GI-15 in ChrII in *B. melitensis* bv1, compared to bvs. 2 and 3 (76) is notable to mention. GI-15 contains 7 ORFs, which encode homologs to the BRUAB_RS13255 (ex BruAb2_0591; Twin-arginine translocation pathway signal sequence domain-containing protein); BR_RS13010 (Lrp/AsnC family of DNA binding transcriptional regulator), BRUAB_RS13270 and BRUAB_RS13275 (ex BruAb2_0594 and 0595, respectively; ABC transporter substrate-binding proteins) and BRUAB_RS13280 (ex BruAb2_0596; FAD binding oxidoreductase). More research is needed to identify the role of the GI-15 in *B. melitensis* virulence.

TABLE 2 | Comparative genetic studies including *B. melitensis* and *B. ovis*.

Strains	Methods	<i>B. ovis</i> genome with respect to <i>B. melitensis</i> .	References
<i>B. ovis</i> REO198; <i>B. melitensis</i> 16M	Whole-genome hybridization	It lacks 80 ORFs distributed in Gls 1, 2, 5, 7, and 9.	(75)
<i>B. ovis</i> ATCC25840; <i>B. melitensis</i> 16M	Sequencing of <i>B. ovis</i> genome and comparison with reference genomes	Evidences of genome degradation. It contains 264 unique and 539 missing genes.	(43)
<i>B. melitensis</i> 16M and ATCC 23457; <i>B. ovis</i> ATCC25840	Identification of TCS coding genes in available genomes	There are four inactivated TCS genes (<i>prfS</i> , <i>tceS</i> , <i>moaR</i> and <i>stcA</i>)	(77)
<i>B. melitensis</i> 16M, 63/9 and Ether; <i>B. ovis</i> 63/290	DNA microarray	<i>B. ovis</i> lacks Gls 1, 2, 6, 11, 13 (<i>B. melitensis</i> lacks Gl 14; and BME_bv1 also lacks Gl15)	(76)

KNOWN AND UNDEFINED MECHANISMS OF *B. MELITENSIS* AND *B. OVIS* VIRULENCE FACTORS MAY EXPLAIN DIFFERENT PATHOPHYSIOLOGICAL CONSEQUENCES

Both *B. melitensis* and *B. ovis* show similar ability to invade, survive and replicate inside professional and non-professional phagocytic cells from their natural hosts as well as other hosts (81–83), but their pathophysiological consequences are quite different. Contrary to many bacteria, but similar to other species of the genus *Brucella*, they don't produce or carry any classical virulence factors, such as toxins, pili and or fimbriae, capsule, drug-resistant forms or antigenic variations. Instead, they use several atypical virulence factors to stealthily enter cells, evade intracellular killing and hamper host immune responses (84).

To date, fewer than 200 gene products have been identified as *Brucella* virulence factors (85), although only some of them have been extensively studied, with much more detail in *B. melitensis* than in *B. ovis*. Among them, LPS is the most broadly studied, since its composition modulates a distinctive phenotype between these two *Brucella* species, but it does not seem to affect its virulence. The overall structure of the lipid A and the core oligosaccharide of both LPS species is presumed to be similar, while the lack or the presence of the terminal O-polysaccharide (O-PS) is associated with the rough or smooth phenotypes of the *B. ovis* or *B. melitensis*, respectively. Independently of this major difference, both LPSs are involved in reduced endotoxic activity, low proinflammatory cytokine production, immune system evasion, cell invasion and resistance to complement and antimicrobial peptides destruction, although some of these properties in *B. ovis* are in association with outer membrane protein (OMP) expression pattern (86–90).

OMPs are other well-known virulence factors involved not only in the *Brucella* outer membrane stability but also in the host: pathogen initial interaction and host cell function modulation (91, 92). Among them, a family of highly conserved 25kDa OMPs is one of the most studied in the *Brucella* genus. *B. ovis* Omp25 presents a 36 bp deletion with respect to *B.*

melitensis that affects a surface-exposed loop of the protein and might influence the *B. ovis*: host interaction (93). Despite this difference, *B. melitensis* and *B. ovis* Omp25 deletion mutants have shown to be attenuated in mice and pregnant goats, and were considered good vaccine candidates (94, 95). Another 25 kDa OMP family difference between these two species of *Brucella* is the absence of the Omp25b encoded gene in the genome of *B. ovis*, although this protein does not seem to be essential for the establishment of *Brucella* spp. (93). Contrarily, Omp25d and Omp22 play critical roles in the entry of *B. ovis* into mammalian cells, but there is no evidence that they perform this function in *B. melitensis* (82, 96). Many other differences exist between *B. melitensis* and *B. ovis* OMPs and their participation in differential pathogenicity and host preference has been predicted *in silico* or tested *in vitro*, but not by *in vivo* challenge of the preferred host (43, 82, 86, 97, 98).

The two-component system (TCS) *BvrR/BvrS* is an experimentally demonstrated virulence factor in *B. abortus* that not only modulates the homeostasis of the outer membrane but also influence metabolic pathways and *Brucella* adaptation to the intracellular environment (99). The *bvrR/S* genes of *B. melitensis* have a high degree of identity with those from *B. abortus* with only four different amino acids in each sequence, and putative *bvrR* and *bvrS* genes exist in *B. ovis* genome (100). While *B. melitensis* *BvrR* mutants are defective in the invasion of Hela cells (101), *B. ovis* *BvrR/S* mutants couldn't be generated suggesting that besides the multiple functions attribute to this TCS in smooth *Brucella* strains, would also be necessary for the *in vitro* free survival of *B. ovis* (102).

Similar, *VjbR* is a global transcriptional activator of virulence factors. Both *B. melitensis* and *B. ovis* *vjbR* mutants have been reported to have the same level of internalization but may be severely impaired to survive in macrophages and human trophoblastic cell line compared to WT strains (101–104). Consistently, both *vjbR* mutants are cleared from the spleens of infected mice few weeks p.i. (102, 103) and *B. melitensis* Δ *vjbR* is safe in its natural host (36, 105). In *B. melitensis*, *VjbR* regulates the positive transcription of two of the main virulence factors, the type four secretion system (T4SS) encoded

by the *virB* operon, and flagellar genes, during vegetative growth and intracellular survival (101). Apparently, VjbR also regulates the *virB* expression in *B. ovis*, but not flagellar genes (102). Moreover, the flagellar loci seem to be dispensable for *B. ovis* virulence in mice and their expression have not been confirmed to date (106), though some flagellar proteins are expressed in and required for *B. melitensis* virulence in mice and goats (90, 107).

The T4SS is a translocator of effector molecules that allow *Brucella* to survive intracellularly and establishes its replicative niche. Its essential role has been experimentally demonstrated in both *B. ovis* and *B. melitensis* (90, 108–110), although a different regulatory mechanism was observed. The VirB operon is highly expressed under pH neutral culture conditions in *B. ovis* and *B. melitensis* 16M, and is further induced under acidic culture conditions in *B. melitensis* but not in *B. ovis* (111, 112). In addition, the number and identity of the T4SS translocated effectors, the regulation of their expression and the way they modulate the outcome of the infection in both *Brucella* species remains to be elucidated. One example worth mentioning is TcpB (also called BtpA or Btp1), a TIR-domain containing protein, which targets TIRAP and represses pro-inflammatory cytokine production by TLR2- and TLR4-signaling pathways (113). The *tcpB* gene is well-conserved in the *B. ovis* genome (43), but little is known about its expression and function during *B. ovis* infection.

Another potential difference in virulence factors is the function of the cyclic B-1,2-D- glucans (C β Gs) in smooth and rough natural *Brucella* species. C β G are polysaccharides present in the periplasmic space of *Brucella* that play an important role in environmental sensing, osmo-adaptation and interaction with the host cell membrane (114). While C β Gs-defective *B. ovis* shows less internalization by macrophages, the microorganism's intracellular survival and replication are not affected (102). *B. abortus* mutants of the C β G synthase (*cgs*) are impaired in intracellular replication, but they do not show defects in internalization (115). The different phenotypes could be in part due to C β G contribution to the architecture and stabilization of the bacterial envelope in rough strains, while in smooth strains it may be required in order to avoid the fusion of the lysosomes with the *Brucella*-containing vacuole. At the same time, both *cgs* mutants show an attenuated phenotype in the mouse model of infection (102, 116). Although the participation of C β G in *B. melitensis* molecular pathogenesis has not been studied, it is very likely that its function is homologous to that of *B. abortus*.

Another difference is that *B. ovis* express an ABC transporter system (abcEDCBA) which is absent in *B. melitensis* and that appears to compensate for a lack of alternative nutrient import pathways. This ABC transporter plays a key role in the intra- and extracellular survival of *B. ovis* in cell culture and during the early stages of infection in mice and rams (110, 112, 117).

As we emphasize throughout this section, several structural and functional differences between *B. melitensis*' and *B. ovis*' major virulence factors have been identified, yet few of them have been effectively

related to differences in host preference or pathogenesis (Table 3). Undoubtedly, future studies, which consider both the pathogen and the host (cell receptors, molecular mechanisms or environment), will help address these voids of knowledge.

LABORATORY PROCEDURES FOR DIFFERENTIAL DIAGNOSIS OF BRUCELLOSIS IN GOATS AND SHEEP

Laboratory tests are necessary to differentiate brucellosis in small ruminants from other infectious agents commonly causing abortion (*Campylobacter fetus* subsp. *fetus*, *Chlamydomphila abortus*, *Toxoplasma gondii*, *Coxiella burnetti*) and orchio-epididymitis (*Histophilus somni*, *Actinobacillus seminis*). Laboratory tests include either the detection of immune response or identification of the agent. Bacterial isolation remains the gold standard for diagnosis of brucellosis, but due to several drawbacks previously mentioned, other safer, cheaper, and faster methodologies have been developed. Thus, PCR-based methodologies have successfully replaced culture and allowed further characterization of the agent (Table 4). Because of its practicability and its wide use, conventional PCR has become the primary diagnostic option for specific detection of *B. melitensis* and *B. ovis* DNA in biological samples (118, 119). More recently, a more simple and inexpensive technique called loop-mediated isothermal amplification (LAMP) assay has been optimized for *B. melitensis* DNA detection in samples from goats with clinical disease (120). Molecular techniques also allow: (1) semi-quantification of *Brucella* DNA in biological samples (121), (2) genotyping of *B. melitensis* strains (122), and (3) differentiation between *B. melitensis* field strains and vaccine strain Rev.1 (91, 123).

Antibody detection tests remain the most cost-effective approach for the screening and detection of *B. melitensis*-infected herds, especially under resource-limited settings. Since no single serological test is adequate in all epidemiological situations, the simultaneous use of at least two different serological techniques that vary according to epidemiological situation is strongly recommended to evaluate brucellosis status in small ruminant herds (14). Macro-agglutination tests using buffered smooth *Brucella* antigen, such as buffered plate agglutination test (BPAT) or rose Bengal test (RBT), or indirect enzyme-linked immunosorbent assays (iELISAs) are generally employed for screening, with fluorescence-polarized antigen (FPA) or complement fixation (CF) as complementary or confirmatory tests, respectively. To confirm false-positive serological results caused by infection with cross-reacting bacteria (i.e., *Y. enterocolitica* O:9, *E. coli* O157 or *S. urbana*) in *B. melitensis*-free areas, the brucellin skin test (BST) is the preferred diagnostic test (14, 132–134), and the agar gel immunodiffusion (AGID) test is used to serologically differentiate infected from vaccinated goats and sheep in the field (135). For *B. ovis* serological antibody detection, AGID, ELISA and CF are the recommended techniques; however, they commonly show highly variable results with considerable number of false-positive and false-negative

TABLE 3 | Comparison of virulence factors in *B. melitensis* and *B. ovis*.

Virulence factor	Descripción	<i>B. melitensis</i>	<i>B. ovis</i>
LPS	Structural integrity Cell invasion and evasion of host immune response		Similar core and lipid A Similar role in virulence
OMPs	Outer membrane stability Interaction with host cells	Presence of O-PS Omp25 is involved in virulence in goats	<i>wboA/B</i> genes absent Many differences in OMP genes Omp25 is involved in virulence in mice Omp25d and Omp22 are involved in cell internalization
BvrR/BvrS	TCS. Environmental sensing and regulation of gene expression (OMPs, virulence factors)	Essential for cellular invasion	Mutants couldn't be obtained Essential for <i>in vitro</i> cell-free survival?
VjbR	Quorum-sensing transcriptional regulator (VirB, flagellar genes)		Involved in intracellular replication and virulence
T4SS (VirB operon)	Translocator of effector proteins to host cells		Key role in the establishment of <i>Brucella</i> replicative niche Essential for intracellular replication and virulence
CβG	Environmental sensing Osmo-adaptation Interaction with host-cells	Enhanced expression in acidic conditions <i>in vitro</i> Unknown Cgs gene conserved with <i>B. abortus</i> . Essential for intracellular replication?	Highest expression at neutral pH Involved in cell internalization and virulence in mice
ABC Transporter abcEDCBA	Unknown Involved in post-transcriptional regulation of VirB expression	<i>abcEDCBA</i> genes absent	Involved in intracellular replication and virulence in mice and rams
Flagellar proteins	Unknown	Essential for virulence in mice and goats	Non-functional
TcpB	T4SS secreted effector	Involved in TLR2/4 inhibition by TIRAP degradation	Unknown Gene conserved
PriS	Member of TCS PriS/R (proline sensor-regulator)	Necessary for <i>Brucella</i> persistence in mice	Gene absent
TceS	Member of TCS TceS/R	Involved in <i>Brucella</i> intracellular survival and virulence in mice.	Gene absent

TABLE 4 | PCR-based methods for *B. melitensis* and *B. ovis* specific identification.

Level of identification	Methodology	References
<i>B. ovis</i>	Conventional PCR Multiplex PCR	(119, 124, 125)
<i>B. melitensis</i>	Conventional PCR LAMP Real Time (RT)-PCR Immuno-magnetic separation-PCR (IMS-PCR)	(126, 127) (120) (128) (129)
<i>B. melitensis</i> biovars	Multiple-locus variable number tandem repeat analysis (MLVA)	(122)
<i>B. melitensis</i> field strain and <i>B. melitensis</i> Rev.1	PCR-restriction fragment length polymorphism (PCR-RFLP) Duplex PCR Multiplex PCR Single nucleotide polymorphism (SNP)-based test	(91, 123) (130) (127) (131)

serologic reactions (136). The development of a reliable and easy-to-perform test for serological detection of *B. ovis* will be a necessary step for controlling the disease.

PREVENTION AND CONTROL MEASURES

Simple measures to prevent the introduction of the agent in a herd are: (1) to maintain a closed herd, (2) to avoid contact with infected animals and unnecessary visitors, (3) to use separate pens for lambing, and (4) to keep facilities clean and disinfected. If external animals must be incorporated, they should come from an accredited brucellosis-free herd or a herd without history of brucellosis, and the individual serological and clinical negative status must be confirmed before being introduced into the herd. All these measures are easier to implement in intensive farming, but are almost impossible to achieve in extensive, transhumant or nomadic pastoralism situations, which is the way the majority of caprine and ovine production are managed. Thus, surveillance and vaccination, together with imposing livestock movement control, are the main measures aimed at maintaining brucellosis-free flocks in these conditions.

After *B. melitensis* infection, three different options are recommended for control and eradication: (1) test and slaughter of positive animals, (2) massive vaccination, or (3) pre-pubertal female vaccination with test and slaughter of infected animals (18, 137). Although it is not the purpose of this article to describe these options in detail as has been done extensively in previous publications, it is worth noting that much of the success of control and eradication programs is based on reliable laboratory diagnostic results and the use of suitable qualified vaccine lots or seed stock sources used to make the immunogen. The selection of the criteria to be employed depends on, but is not restricted to: (1) the prevalence of brucellosis, (2) the capacities of the National Veterinary Services, (3) the type of animal husbandry, (4) the geography of the area, (5) financial, (6) technical and personnel resources available, and (7) the compliance of the livestock owners, among other issues. On the other hand, the prevalence of *B. ovis* infection can be reduced by the clinical examination and laboratory testing of rams before the breeding season followed by the culling of those with palpable abnormalities or positive laboratory results. Despite some successful experimental attempts, a commercial vaccine to prevent *B. ovis* infection is not yet available (117, 138, 139). The use of antibiotic treatment is not economically or clinically recommended for the control of brucellosis in infected small ruminants, due to the high failure rate, cost and potential problems related to maintaining the coexistence of infected and healthy animals in a flock.

Even though animal vaccination and other measures presented here have contributed significantly to eradicating or reducing the incidence of brucellosis in many geographical areas, the disease has not been controlled in many other regions and is considered, in many cases, a re-emerging disease. A relatively new complementary approach is the identification

and subsequent selection of animals with natural resistance to *Brucella* infection. Original studies found naturally resistant pigs and cows (140). Later on, this phenotypic characteristic was linked to genetic markers. The alleles of the goat genes *SLC11A1* (formerly *NRAMP1*), *PTPRT*, *IRF3*, and *TNF* were recently reported by us associated with the absence of *Brucella* sero-response in Creole crossbreed goats (141–144), and variants in the *MHC-DRB1* loci were associated with brucellosis susceptibility in Chinese Merino sheep (145). Though, it is clear that resistance to natural infection would rarely be controlled by a single gene, more research is still required to include marker-assisted selection for natural resistance to brucellosis in breeding programs as a significant contribution to the prevention of the disease in small ruminant herds.

CONCLUSIONS AND FUTURE APPROACHES

Brucellosis in small ruminants is an ancient disease that has been eradicated in many countries, but still remains endemic in most regions. There is a vast amount of literature explaining successful eradication campaigns in several areas using available tools (i.e., diagnostic tests, vaccines, culling) adjusted to the particular context. Thus, why is small ruminant brucellosis still endemic in some geographic regions? The answer may be that these tools are simply not available, or they are wrongfully used. Undoubtedly, future development of the combination of less complex diagnostic tests, effective immunogens, enhanced response to vaccination, identification of innate resistant animals to *Brucella* infection, and/or other unknown factors will help to control and eradicate brucellosis in domestic sheep and goats. In the meantime, history informs us that control and eradication programs may be successfully implemented employing available tools and appropriate strategies.

AUTHOR CONTRIBUTIONS

CR conceived and designed the outline of the review and reviewed and edited the document. CR, EM, and UR performed the literature search and wrote the original draft. All authors read and approved the latest version of the article.

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