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Bovine brucellosis: A review on background information and perspective

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Abstract
Brucellosis is a highly contagious disease impacting the dairy sector in India as it causes reproductive impairment in the form of abortion storms, retained placentae and infertility. This paper gives an overview of the bovine brucellosis on Indian context including the factors involved in the spread of infection, besides highlighting the need to implement strict surveillance and control measures taking those developed countries as a model in which the disease has been eradicated through improved hygiene, test and slaughter policy, vaccination and monitoring of animal movements. The paper further discusses the background information on the aetiology including the historical overview, microbiological or phenotypic characteristics, taxonomy, antigenic components, pathogenesis and host immunity against *Brucella*. There is a further need to study and understand the differential immune-mediated responses in different *Brucella* spp. as well as hosts to unravel the newer aspects of diagnosis, treatment and vaccine development.

Keywords: aetiology, bovine, brucellosis, immunity, pathogenesis

1. Introduction
Livestock provides a lifeline for a large proportion i.e. 95 per cent of the world's rural population that lives in the developing world and cultivates 64 per cent of the world's arable land [1, 2]. In India, the dairy sector plays a very significant role in the rural economy. In states like Rajasthan, the receding precipitation and changes in the pattern of rainfall distribution is leading the farmers to gradually shift their focus from agriculture to livestock production for their livelihood, but the cattle breeding is losing ground due to the lack of economic viability. Despite India taking the credit of highest milk production in the world i.e.155.5 million tonnes (2015-16) as per Annual Report [3]; its yield continues to remain low at 1.1 tonnes per head during 2010-12 as stated by Reddy and Ramappa [4]. There has been a long term continual drain on the production as well as productivity of the bovine population of India because of endemic infectious diseases.

One of the important diseases impacting the dairy sector in India is brucellosis. The disease has major socioeconomic importance worldwide, especially in developing countries like India where the disease control programmes are either non-existent or inadequate. As per Singh *et al.* [5], the disease is responsible for a loss of Rs. 442.24 per cattle and Rs. 1183.65 per buffalo in India. Radostits *et al.* [6] has attributed the economic burden posed by bovine brucellosis to: the abortion storms in newly infected herds, a high level of retained placentae and hence endometritis or metritis resulting in reduced milk production, infertility.

Besides being a threat to the livestock, brucellosis has been recognised by OIE as the second most important zoonotic disease in the world after rabies. The *Brucella* species, particularly *Brucella melitensis* and *Brucella suis* are potential agents of biological terrorism [7, 8]. The World Health Organization (WHO) laboratory bio-safety manual classifies *Brucella* in risk group III [9]. The disease is a serious occupational hazard for humans, and has been found to be associated with farm workers, veterinarians, veterinary pharmacists, animal attendants, abattoir workers and laboratory attendants as evidenced by Young [10].

As per Singh *et al.* [5], many factors are responsible for the spread of brucellosis among livestock in India, such as, absence of a control policy, failure to vaccinate young female calves, non-implementation of test and slaughter, ban on cow slaughter in many Indian states, absence of treatment regimen and usual practice of selling positive reactor animals to other farmers. Other risk factors include poor farm hygiene, unrestricted trade and movement of animals, use of local cattle yards and fairs for trading, the practice of returning non-lactating

animals to villages for seasonal maintenance, and the use of semen from infected bulls of unknown health 1status for artificial insemination [11]. According to one report, large herd size enhances the exposure potential, especially following abortions, through increased contact and common feeding and watering points promoting transmission of *Brucella* organisms [12].

Data from India are sparse, but with the largest livestock population in the world and no brucellosis control program in place, millions of *Brucella* positive animals are likely to present [13]. India needs to implement the brucellosis surveillance and control model from the developed countries which have controlled the disease through strict and scrupulous control regimens including improved hygiene, test and slaughter policy, vaccination and monitoring of animal movements [14-16]; thus highlighting the importance of diagnosis and vaccination oriented research on brucellosis.

2. Bovine brucellosis

Brucellosis in cattle occurs worldwide, except in countries where it has been eradicated, including Britain, Norway, Sweden, Finland, Denmark, Germany, Belgium, Netherlands, Switzerland, Austria, Czech Republic, Slovakia, New Zealand, Canada, France and Italy. However, the disease is an important issue in developing countries, with biogroups of *B. abortus* usually occurring particularly in the tropical countries [17]. Historically, in the Indian subcontinent, the credit of first investigation of contagious abortion in livestock, associated with brucellosis, goes to the Imperial Veterinary Research Institute (now Indian Veterinary Research Institute), Mukteshwar, in northern India [18].

Brucellosis in cattle is usually caused by biovars of *Brucella abortus*. In those areas where cattle are kept in close association with sheep or goats, infection can also be caused by *B. melitensis* [19]. Occasionally, *B. suis* may cause a chronic infection of the mammary gland of cattle, but it has not been reported to cause abortion or spread to other animals [20]. Brucellosis has also been reported in the domestic buffaloes, American and European bison, yak, elk and camel. The manifestation of brucellosis in these animals is similar to those in cattle [21]. In India, there was not much work done in buffaloes [22]. Worldwide, *B. abortus* biotype 1 is the most common among nine biotypes of the organism causing brucellosis in cattle. However, *B. abortus* biotype 3 tends to predominate in indigenous cattle population in Africa and Asia. In India, cattle and buffaloes harbor predominantly *B. abortus* biotype 1 infection [23] and exceptionally *B. abortus* biotype 3 [24]. On the contrary, Mohanty and Panda [25] identified distribution of *B. abortus* biotypes 1, 3, 6, 7 and 9, with biotype 3 being dominant in cattle.

In dairy cattle, infection occurs in all ages but most commonly in sexually mature animals. Mostly, abortions occur in unvaccinated heifers after the 5th month of pregnancy [26]. The disease is usually asymptomatic in young animals and non-pregnant females. Following infection with *B. abortus* or *B. melitensis*, pregnant adult females develop a placentitis usually resulting in abortion between the fifth and ninth month of pregnancy. Even in the absence of abortion, profuse excretion of the organism occurs in the placenta, fetal fluids and vaginal discharges. The mammary gland and associated lymph nodes may also be infected, and organisms may be excreted in the milk. Subsequent pregnancies are usually carried to term, but uterine and mammary infection recurs, with reduced numbers of organisms in afterbirth products and milk. Adult male cattle may develop orchitis/epididymitis and brucellosis may be a cause of infertility in both sexes. Hygromas, usually involving leg joints, are a common manifestation of brucellosis in some tropical countries [27].

Cattle are the main reservoir of *B. abortus* and the introduction of pregnant, recently aborted, or recently calved animals with brucellosis from infected herds are the main source of infection for clean herds. Aborted foetuses as well as fetal membranes and uterine secretions eliminated after abortion or parturition are the most important sources of infection [28]. *Brucella* may retain infectivity for several months in water, aborted foetuses and foetal membranes, faeces and liquid manure, wool, hay, on buildings, equipment and clothes [29]. The disease can also be transmitted to calves vertically [30] and through contaminated milk [31, 32]. *B. abortus* is excreted in bovine milk and can remain viable in milk, water and damp soil for up to 4 months [33]. Venereal transmission is not a major route of infection under natural conditions, but artificial insemination with contaminated semen is a potential source of infection [34].

Although infection may occur through the skin, conjunctiva or respiratory mucosa by inhalation [35, 36], the most common route of infection in cattle is the gastrointestinal tract [35, 37], from where the infection spreads to local lymph nodes where *Brucella* replicates intracellularly in phagocytes [38]. Invasion of lymphatic vessels is followed by bacteraemia leading to systemic infection, favouring colonisation of the pregnant uterus, male genital organs, and mammary gland [36]. *B. abortus* has a strong tropism to the uterus during the last trimester of gestation, which is thought to be due to high concentrations of erythritol and steroid hormones [39]. However, *Brucella* has also been found in the reproductive tract of animals with no detectable levels of erythritol, the role of this sugar in the virulence of the organism has been put into question [40]. *B. abortus* Strain 19 is spontaneous attenuated mutant widely used to vaccinate cattle. S19 is the only *B. abortus* strain that is inhibited by erythritol [40].

3. Historical Overview

Archaeological and anthropological studies have confirmed that brucellosis has been present in humans and animals since ancient times. Moreno *et al*. [41] reported the presence of *Brucella abortus* and *Brucella melitensis* in double-hoofed animals around twenty million years ago in their study. Examination of the ancient Egyptian bones, dating back to around 750 BC, showed evidence of sacroiliitis and other osteoarticular lesions, common complications of brucellosis [42] and examination of the skeletal remains of the Roman residents of Herculaneum (Naples, Italy) killed by the catastrophic volcanic eruption of Mt. Vesuvius in the late August, 79 AD revealed vertebral bone lesions typical of brucellosis in more than 17% of the residents [43].

The seminal discovery of the causative agent of brucellosis, "*Micrococcus melitensis*" (later named *Brucella melitensis*), by the British Surgeon Captain David Bruce, his wife Mary Elizabeth Steele and the Maltese microbiologist doctor Giuseppe Caruana-Scicluna has been eagerly described in many assays [44-47]. Ten years after the isolation of *M. melitensis*, the Danish scientist Bernhard Bang identified "*Bacillus abortus*" (later named *Brucella abortus*) in bovine aborted foetuses [48]. Traum [49] reported the isolation of another organism related to *M. melitensis* (later assigned as *Brucella suis*) from aborted pigs in United States.

American microbiologist Alice Catherine Evans accomplished the final link of these zoonotic bacteria [50] thus helping outstandingly in understanding the epidemiology of brucellosis and the founding of milk pasteurization as a preventive measure. Then, in 1920, Louis Meyer and Wilbur Shaw honoured David Bruce and proposed to group these pathogenic bacteria within a single genus named *Brucella* [51]. The events that followed all these inspiring investigations have demonstrated the existence of different *Brucella* species that cause brucellosis in domestic animals (cows, sheep, goats, pigs, camels, reindeer, and dogs), wild land animals (bison, elk, hares, muskox, caribou, foxes, and several rodents) and sea mammals (dolphin, whales, seals, and walruses) [52, 53].

4. Microbiological/ Phenotypic Characteristics

Brucella species are facultative intracellular, gram negative bacteria that lack capsules, flagella, and endospores [11]. They are either coccobacilli or short bacilli with a size range of 0.5-0.7 µm wide by 0.6-1.5 µm long [54]. They can occur singly, in groups, or in chains, and grow well on media containing blood or serum [55]. The organism is not acid-fast but does resist decolourization by weak acids and thus stains red with Stamp's modification of the Ziehl-Neelsen stain [56-58]. *Brucella* spp. are slow growers and their growth is often improved by carbon dioxide which is essential for some strains. Most wild strains of *B. abortus* are fastidious and slow-growing, and require carbon dioxide (5 to 10 per cent) supplementation for primary isolation at an optimal growth temperature of 36-38°C, while growth of *B. melitensis* is not dependent on an atmosphere of 5 to 10 per cent of CO_2, although there might be some exceptions [59].

On suitable solid media, *Brucella* colonies are visible after 2 days and are 0.5 to 1.0 mm in diameter with a convex and circular outline; smooth strains are transparent and pale yellow while rough colonies are more opaque with a granular surface [60]. *Brucella abortus, B. melitensis, B. suis* and *B. neotomae* may occur as either smooth or rough strains expressing smooth lipopolysaccharide (S-LPS) or rough lipopolysaccharide (R-LPS) as major surface antigens, while *B. ovis* and *B. canis* are naturally rough strains [61]. *Brucella* species are positive for catalase, oxidase and urease. The metabolism of the *Brucella* is mainly oxidative and they show little action on carbohydrates in conventional media [62]. The guanine-plus-cytosine content of the DNA is 55-58 moles/cm. No *Brucella* species has been found to harbor plasmids naturally although they readily accept broad-host-range plasmids [29].

5. Taxonomy

The genus *Brucella* belongs to the family *Brucellaceae* within the order *Rhizobiales* of the class Alphaproteobacteria [52]. The class Alphaproteobacteria includes organisms that are either mammalian or plant pathogens or symbionts. Within the family *Brucellaceae*, *Ochrobactrum* is the closest phylogenetic neighbour of *Brucella*. Species and biotypes classification of *Brucella* is historically based on natural host preference and phenotypic traits [62]. Currently *Brucella* comprises ten species which include the six classical *Brucella* species, *B. melitensis*, biotypes 1-3 (sheep and goats); *B. abortus*, biotypes 1-7 and 9 (cattle and other Bovidae); *B. suis* biotypes 1-5 (biotypes 1-3 pigs, biotype 4 reindeer, biotype 5 small rodents); *B. canis* (dog); *B. ovis* (sheep) and *B. neotomae* (desert wood rats).

The DNA-DNA hybridization studies showed that, according to the common taxonomic rules (DNA homology >70%, the classical species only represent one species [19] and therefore should be combined into the single genomospecies *Brucella melitensis.* Nevertheless, to avoid confusion, the "Subcommittee on the taxonomy of *Brucella"* proposed to keep the nomen-species.

Further, three novel species have been added to the genus, *B. pinnipedialis* (seals), *B. ceti* (dolphins and whales), and *B. microti* (common vole, red foxes and also from soil). Most recently *B. inopinata* isolated from a breast implant wound has been described as a new species with so far unknown animal reservoir [62]. There are two other isolates, with typical *Brucella* characteristics but distinct from the currently described species, known to have caused individual incidences of diseases. These isolates are still awaiting final taxonomical classification, one being referred to as Baboon type in the meantime [63].

6. Antigenic Components

The outer cell membrane resembles that of other Gram-negative bacilli with a dominant lipopolysaccharide component which is considered the target for many serological and immunological studies and the principal virulence factor of *Brucella* [64]. All *Brucella* species, except *Brucella ovis* and *Brucella canis*, contain smooth lipopolysaccharide (S-LPS) in their outer cell wall [60]. Strains with S-LPS are more virulent and more resistant to intracellular destruction by polymorphonuclear leukocytes than the strains with rough lipopolysaccharide [64].

The S-LPS exist as antigenic epitopes A and M which have different quantitative distribution among the smooth *Brucella* strains and are absent, in the rough *Brucella* strains. This is of value in differentiating biotypes of the major species using absorbed monospecific A and M antisera [65]. Wilson and Miles [66] reported that A antigen is associated with *B. abortus* (A-dominant) and M antigen is associated with *B. melitensis* (M-dominant). Outer membrane structural proteins (Omp25) are also useful in diagnostic tests. Others, such as ribosomal proteins (L7/L12) and fusion proteins, have demonstrated a protective effect against *Brucella* based on antibody and cell mediated responses [67].

7. *Brucella* pathogenesis and Host immunity

Brucellae have a predilection for macrophages, dendritic cells (DCs) and trophoblasts [68] and the bacteria can enter, survive, and replicate within these cells and cause disease [69]. *Brucella* gain access to the host through inhalation, conjunctiva, skin abrasions and ingestion [70]. *Brucella* spp. can invade epithelial cells of the host, allowing infection through mucosal surfaces: M cells in the intestine have been identified as a portal of entry for *Brucella* spp. [71]. Trophoblasts are the placental cells that are targeted during infection of pregnant cows. Although it is a fastidious bacterium, *Brucella abortus* does have major biosynthetic pathways [72] available to it. In its primary host, cattle, the metabolic pathway for the breakdown of erythritol is one that is most desirable, it is even used "preferentially to glucose" [73]. Since erythritol is found in bovine placenta, this may be a possible factor in the bacteria's virulence. As per Preez and Malan [74], about 90% of infected cows remain chronic and may remain infected for the rest of their lives, with the bacteria being localised in the udder tissue or lymph nodes.

Once *Brucella* spp. have invaded, usually through the

digestive or respiratory tract, they are capable of surviving intracellularly within phagocytic or non-phagocytic host cells [39]. *Brucella* has the ability to interfere with intracellular trafficking, preventing fusion of the *Brucella*-containing vacuole (BCV) with lysosome markers, and directing the vacuole towards a compartment that has rough endoplasmic reticulum (RER), which is highly permissive to intracellular replication of *Brucella* [38,75,76]. Interestingly, invasion through the digestive tract does not elicit any inflammatory response from the host [77]. Therefore, *Brucella* spp. invades silently or unnoticed by the innate immune system of the host. In fact, *Brucella* spp. have mechanisms that prevent activation of the host innate immune system [78]. Indeed, *Brucella* Toll/interleukin-1 receptor (TIR) domain-containing protein prevents Toll-like receptor (TLR) 2 signalling by interfering with MyD88, and also inhibits DC maturation, cytokine secretion and antigen presentation [79, 80]. *B. abortus* also induces suppression of the transcription of pro-inflammatory mediators in trophoblastic cells at very early stages of infection [81].

Brucella lacks well-known bacterial virulence factors such as cytolysins, capsules, exotoxins, secreted proteases, fimbriae, phage-encoded toxins, and virulence plasmids [82, 83]. The brucellae infect phagocytic macrophages and non-phagocytic epithelial cells (e.g., HeLa cells) *in vivo* and *in vitro* [36, 84, 85]. *Brucella* virulence relies on the ability to survive and replicate in the vacuolar phagocytic compartments of macrophages. Many *Brucella* virulent factors, such as lipopolysaccharide (LPS), type IV secretion system i.e. T4SS [86, 87], and the BvrR/BvrS two-component system [88], have been identified to be critical in the intracellular process of *Brucella* inside macrophages [89]. The clinical manifestations of brucellosis may not be mediated by these virulence factors, but they are critical for *Brucella* to survive and replicate inside host cells.

While prolonged persistence of the brucellae in macrophages leads to the chronic infection, extensive replication of the bacteria in placental trophoblasts results in acute reproductive tract pathology and abortion in natural hosts [90]. Specifically, the *Brucella* lifecycle contains two phases: (i) chronic infection of phagocytic macrophage leading to *Brucella* survival and replication, and (ii) acute infection of non-phagocytic epithelial cells leading to reproductive tract pathology and abortion. Spleen and liver contain many bacterial cells after *Brucella* invasion. After a majority of *Brucella* cells are killed *in vivo*, the remaining *Brucella* cells will persist and live for a long time *in vivo* [91].

Brucellosis has long been acknowledged as a model to study the immunity against intracellular bacterial infections. Although antibodies specific for the O-antigen or O polysaccharide of the LPS can confer partial protection in some host species, cell-mediated immunity (CMI) plays a very critical role in protection against virulent *Brucella* infection. For the first time, in 1958, Holland and Pickett demonstrated that *Brucella* spp. extensively replicated inside murine macrophages in a 'silent mode', without generating toxic effects [92]. Later, Mackaness confirmed the cellular basis of immunity in brucellosis, suggesting the important role of the interaction between T lymphocytes and macrophages in defense against intracellular pathogens [93]. It is noteworthy that, two decades later, brucellosis was again used as the model infection associated with interferon-γ (IFN-γ) production in the description of the Th1/Th2 dichotomy concept by Mosmann *et al.* [94]. The maturation and proinflammatory production of cytokines of dendritic cells is critical for controlling *Brucella* infections [95].

Recently it was found that *B. abortus* vaccine strain RB51 and *B. suis* vaccine candidate VTRS1 induce caspase-2-mediated apoptotic and necrotic macrophage cell death [96, 97]. The virulent *Brucella* strains inhibit the programmed cell death. Caspase-2-mediated cell death induced by vaccine strain RB51 may promote an effective *Brucella* antigen presentation by a cross-priming mechanism [96, 98]. Passive transfer assays with mice suggest that both $CD4^+$and $CD8^+$ T cells are important in protective immunity against brucellosis [99, 100]. To confer protection against *B. abortus* infection, immune $CD4^+$ T cells secrete many cytokines, including IFN-γ that stimulates the antimicrobial activity of macrophages [101-103]. A crucial role of IFN-γ in the resistance to *Brucella* infection was demonstrated in mice by *in vivo* antibody neutralization experiments [102] and an IFN-γ knockout mouse study [104]. $CD8^+$ cytotoxic T lymphocytes (CTL) are critical in killing *Brucella*-infected target cells [103, 105].

Cross-talk between the host immune system and *Brucella* results in either the eradication of the pathogen, or the development of intracellular parasitism and establishment of chronic disease. Host protection against *Brucella* depends on cell-mediated immunity, involving mainly activated professional APCs, Th1 cells, and CD8+ CTLs [106]. On the other hand, *Brucella* has developed various strategies to evade innate and adaptive immune responses, aimed at the establishment of an intracellular niche for longterm survival and replication [107-109]. It should be mentioned that immune response mechanisms to brucellosis may diverge, and they are dependent on the host, and the species or strain of *Brucella* [109, 110].

8. Conclusions

Besides the implementation of strict surveillance and control measures, there is a further need to study and understand the pathogenesis and differential immune-mediated responses in different *Brucella* spp. to unravel the newer aspects of immunodiagnosis, immunotherapy as well as vaccinology; which could aid in eradication of the disease.

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