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Bubaline Brucellosis

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Brucellosis causes serious economic losses and is an important zoonosis [1-5]. Buffaloes in many countries are known to be affected with *Brucella abortus* [6-8] and less frequently with *Brucella melitensis* [9,10]. Similar to cattle Brucella infections are known to result in late gestation (6-9 months) abortions [11-14], infertility [15-17] and latent infection of mammary gland lymph nodes with shedding of organisms in the milk [10], yet abortions are less common in buffaloes [4] with the disease being endemic in most buffalo raising countries. Shedding of Brucella in milk creates a potential threat to human health particularly for consumers using unpasteurized milk and milk products [10]. Diagnostic evaluations of Brucella infections in buffalo have utilized approaches employed in cattle with nearly similar or a slightly lower efficiency [18,19]. Isolates of Brucella from water buffalo were less virulent compared to those from cattle [19] suggesting some degree of resistance in buffalo towards *Brucella abortus*. Even in buffalo herds heavily infected with *Brucella abortus*, 20% of the animals remain negative by serologic tests and presumably uninfected at all times [20]. Identification of specific genotypes (Nramp11 BB) amongst buffalo populations [21] with resistance towards *Brucella abortus* infection have not only confirmed the presumptive lower morbidity with *Brucella abortus* in buffalo against cattle but have also offered opportunities to control this disease by genetic selection. A slightly lower incidence of brucellosis has been recorded in buffaloes compared to cattle in studies that simultaneously evaluated the serologic presence of brucellosis in these two species [15,22-24], however, in other studies a higher incidence of the disease was recorded in buffaloes compared to cattle [25-27]. Thus, it can be presumed that buffaloes are differentially affected with *Brucella abortus*. The preventive measures for eradication or control of the disease in buffalo raising countries are similar to those employed in cattle and there has been increased reporting of the disease during the last few years. In this chapter, the history, etiologic agents, diagnosis, distribution, epidemiology, prevalence, pathogenesis, necropsy findings, clinical signs, immune response, sampling, prophylaxis and zoonoses of brucellosis in buffalo are mentioned.

1. History

Early indications of Brucellosis date back to the Crimean War (1853-1856) in which *Brucella* spp. was linked as the causative agent of human disease [28]. It was first described in 1859 on the island of Malta by Marston. The first identification of *Brucella* spp. was performed by Dr. Bruce in 1887 and in 1897 Dr. Bang identified *Brucella abortus*. Brucellosis is an infectious disease that can affect both wild and domestic animals and humans, caused by several species of the genus Brucella [1,3,5]. Six species are currently known; *B. abortus*, *B. mellitensis*, *B. suis*, *B. canis*, *B. ovis* and *B. neotomae*, which can be distinguished by host specificity or differential characteristics of microbes. In addition, *B. pinnipediae* and *B. cetacae* are being tested as new species [29]. Because of its global expansion, *B. abortus* infection takes different names as Bang's disease, Malta fever or undulant fever.

The first report on the occurrence of brucellosis in buffaloes appears to have originated in India in 1918 at the Indian Veterinary Research Institute, Mukteshwar [30]. Later on brucellosis was detected in Egyptian buffaloes [31] and in 1942, *Brucella abortus* organisms were isolated from buffaloes in India [32], however, abortions caused by *Brucella abortus* appear to be first recorded in India in 1964 [33] and the disease was described in 1968 [6]. In Pakistan the reports on the occurrence of brucellosis appeared in 1967-1968 [34,35]. Many reports on the seroprevalence of the disease have appeared from many countries thereafter including India [12,13,15,22,36-39], Pakistan [8,24,40-46], Egypt [7,47,48], Iran [27], Iraq [49-51], Bangladesh [26,52,53], Vietnam [11], Sri Lanka [54-56], Argentina [57-59], Brazil [60-63], Mexico [64], Trinidad [18,19,65,66] Italy [9,67], Colombia [68], Venezuela [69], and Turkey [70].

2. Etiologic Agent

Bacteria of genus Brucella are non-motile, non-encapsulated, non-spore forming, gram-negative small bacilli (0.6-1.5 μm in length and 0.5-0.7 μm in diameter) which grow in isolation, in pairs or in small groups. Most species are catalase

positive and *B. neotomae* oxidase negative, and they are strict aerobes except *B. ovis* and *B. abortus* which are microaerobic, needing 5-10% CO_2 for development [71]. From the morphology of colonies growing on solid media, they can be classified as smooth (S) or rough (R), the differentiating factor in these colonies is given by the expression of lipopolysaccharides (LPS). LPS are constituted by lipid A, an oligosaccharide (core) and O-polysaccharide, and these components confer genetic, biochemical and biological differences to each *Brucella* spp. The causative agent of brucellosis in buffaloes is *B. abortus*, which affect both draught animals and dairy breeds [6] manifested chiefly by abortions during late pregnancy similar to those observed in cattle [33]. *B. mellitensis* has also been described as a causative agent of brucellosis in buffaloes and cattle [7,72] the biovar 3 being one of the most frequently detected [9]. The risk of inter species transmission of *Brucella* spp. is higher in countries with mixed production systems, as buffaloes-cattle or sheep-goats systems [74].

3. Diagnosis

For the diagnosis of *B. abortus* there are direct techniques that detect the agent or indirect techniques that identify antibodies generated by exposure to the bacteria. Since Brucella microbes are Gram negative, staining can be used in smear samples of organs or body fluids with the Ziehl-Neelsen stain modified by Stamp, which provides insight of microorganisms colored in red on a blue background (Fig. 1).

Figure 1. Smear observation of Brucella spp. with coloring Stamp. - To view this image in full size go to the IVIS website at www.ivis.org . -

Isolation and bacterial identification is an unequivocal diagnosis, samples are preferably taken from abortion materials [74] but isolation can be attempted from milk, colostrum or other tissue samples [9]. To optimize the bacterial isolation, it is convenient to work with a specific culture media (Fig. 2) as agar dextrose, agar potato or Farrell medium, which meet the necessary nutritional requirements for bacterial growth. The latter also has specific antibiotics for Gram negatives, which decrease the growth of other microbes. Cultures should be maintained at 37°C with an enriched atmosphere with 5-10% CO_2 [75].

Figure 2. Observation of *Brucella* spp. in culture media (Skyrrow). - To view this image in full size go to the IVIS website at www.ivis.org . -

The diagnosis through Polymerase Chain Reaction (PCR) allows not only to detect positive samples for *B. abortus* but also to identify more quickly than with conventional (biochemical) techniques the nine biotypes of *B. abortus* described so far, and some vaccine strains [76-78]. Using the PCR technique, Martinez et al. [79] were able to differentiate *B. abortus* biotype 5 from S19, in blood samples of buffaloes which were positive to a complement fixation test.
The serological techniques used are based on the detection of IgM, IgG or both, the most widespread being the Rose Bengal test (RBT) [8,80,81] and Buffered Plate Agglutination Test (BPAT). Serum Agglutination test (SAT), complement fixation test (CFT) [18,80], Enzyme-linked immunosorbent Assays (i-ELISA and c-ELISA) [8,24,61,82,83] and Fluorescence Polarization Assay (FPA) [84,85] are also in use. RBT and BPAT techniques are used for screening, and CFT, ELISAs and FPA are confirmatory techniques with greater sensitivity and specificity, recognized as prescribed analysis for international trade. SAT has been used effectively for many years in surveillance and control programs for bovine brucellosis, but it is not considered an obligatory or alternative test. Studies with sera from buffaloes have shown that the use of FPA as confirmatory technique can improve the sensitivity and specificity. FPA is a technique of simple execution which also allows for variations in the cut-off points, depending on the epidemiological situation in each country or region [84]. The most widely used techniques in milk samples are Milk Enzyme-linked immunosorbent Assays (I-ELISA) and Milk ring test (MRT), which can be used for samples directly from milk tanks. I-ELISA is more sensitive and specific than MRT, but MRT is more used because of its practicality. For both tests, when a sample is positive, all females which contributed to that milk tank must be confirmed individually with serological techniques [86,87]. A combination of culture and PCR techniques has been suggested to be more useful for the diagnosis of *Brucella* sp. in buffalo milk [88]. Some studies showed that results of BPAT technique are highly comparable between bovine and buffalo samples. Using c-ELISA there are differences in the percentage of positive and negative samples, this variation mainly depending on the cut-off used [89]. While these techniques were developed primarily for the diagnosis and control of brucellosis in cattle, they are currently used for samples from buffaloes with previous validation studies and epidemiological evaluations [83,90].

4. Distribution

Brucellosis caused by several species is distributed worldwide [3]. Brucellosis appears to be endemic in buffalo raising countries including India, Pakistan, Egypt, Sri Lanka and possibly many more countries. Some European buffalo raising countries such as Bulgaria appear to be free from brucellosis [3]. Similarly Australia where buffaloes are found in significant numbers is known to be in the list of officially Brucella free countries [3]. The presence of Brucella in buffaloes in many South American and Mediterranean countries continues to be documented in spite of many attempts to control the disease in cattle and water buffaloes. In many developing nations, brucellosis continues to be a problem due to the lack of systematic approaches for diagnosis and control.

5. Epidemiology

Like most infectious diseases, three factors are needed for transmission of *B. abortus*; a susceptible host, the causative agent and the proper environment. In water buffaloes, one of the main sources of infection is fluids expelled during abortion or apparently normal deliveries, which contain high concentrations of bacteria [6]. Large amounts of bacteria are shed and contaminate grass and water [12], which is used by buffaloes not only for drinking but also for congregation [32] and thermoregulation.

6. Prevalence

The prevalence of this disease varies in the different countries or regions where it has been studied. Main factors involved in this variation are the number of animals tested and the serological techniques used for diagnosis. The prevalence in various countries varies from 3% to 20% (Table 1).

Table 1. Prevalence of Brucellosis in Different Countries

Country-region	N samples	N herds	Sample type	Technique	Prevalence	Reference
Brazil	199	-	Serum	RBT	17,31%	Costa et al., 1973 [60]
	462	-	Serum	CFT	10.39%	Mathias et al., 1998 [91]
Colombia	133	3	Serum	RBT ELISA-c	13% 3%	Calderón et al., 2010 [68]
India	-	52	Serum	ELISA	13.4%	Dhand et al., 2005 [28]
	167	-	Serum	SAT	9.4-11.4%	Mehra et al., 2000 [93]
	9456	14	Serum	ELISA	3%	Renukaradhya et al., 2002 [94]
	7153	23 States	Serum	RBPT SAT	1.8%	Isloor et al., 1998 [22]
México	99	3	Serum	RBT Rivanol	13% 7%	Suazo-Cortez et al., 2012 [64]
Egypt	1237	-	Serum	BPTA RBT SAT Rivanol	4.11% 3.52% 3.44% 3.37%	Samaha et al., 2008 [7]
	46	-	Milk	ELISA-i	15.5%	Holt et al., 2011 [73]
	173	40	Milk	ELISA-i	12%	Hegazy et al., 2011 [95]
Sri Lanka	840	-	Serum	ELISA-i	4.2%	Silva et al. 2000 [55]
Iran	400	-	Serum	RBT SAT 2-ME	20.5% 19.5% 11%	Nowroozi-Asl et al., 2007 [96]

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Country-region	**N samples**	**N herds**	**Sample type**	**Technique**	**Prevalence**	**Reference**
Pakistan	704	6	Serum	RBT SAT	15.3-35.4 % 2.9-23.7%	Nasir et al., 2004 [25]
	650	-	Serum	RBT ELISA	9.3% 6.9%	Hussain et al., 2008 [23]
	336	20	Serum	RBT	7.7%	Abubakar et al., 2010 [45]
	800	2	Milk	MRT	3.25%	Ahmad et al., 1990 [42]
	691	-	Serum	SAT MRT	4.5% 8.61%	Maqsood et al., 1988 [40]
	375	-	Serum	RBTP ELISA-i	3.0% 3.2%	Shafee et al., 2012 [24]
	240	-	Serum	ELISA-i SAT	11.25% 10.42%	Zahid et al., 2002 [81]
	1294	-	Serum	ELISA-i RBP	15.2% 6.5%	Munir et al., 2011 [97]
Trinidad	400	4	Serum	SPAT	-	Fostgate et al., 2002 [18]
Iraq	420	-	Milk	MRT	24.2%	Abbas and Al-Deewan, 2009 [50]
	5940	-	Serum	RBT ELISA-i	5.53%	Sharief et al., 2006 [49]
Trinidad	400	4	Serum	SPAT	-	Fostgate et al., 2002 [18]
Venezuela	-	-	Serum	RPAT	10.5%	Francisco and Vargas, 2002 [69]
Vietnam	561	-	Serum	SAT CFT	16.39%	Sharma et al., 1988 [11]
Bangladesh	288	-	Serum	PAT TAT	6.9% 2.4%	Alam et al., 1996 [52]
	105	-	Serum	ELISA-i	2.87%	Rahman et al., 2011 [26]
Italy	-	-	-	-	-	Galiero, 2007

Figure 3. The route of entry and transport of brucella organisms to target sites. - To view this image in full size go to the IVIS website at www.ivis.org . -

7. Pathogenesis

The pathogenesis of *Brucella* spp. has been recently reviewed [98]. The most common infection route in buffaloes is oral, through pastures or water contaminated by traces of abortions or birth fluids of infected buffaloes. Other effective ways are the conjunctiva and skin. The existence of a lesion over the skin or the mucous membrane renders tissues permeable and allows the entry of the bacteria. After infection, *B. abortus* is transported to the local lymph nodes, where it produces lymphoid hyperplasia and an acute inflammatory response. Then it migrates to other lymph nodes, liver and lungs (Fig. 3). In pregnant females, the microbes have high affinity for the uterus and mammary gland. The affinity of *B. abortus* by the

gravid uterus is due to the presence of erythritol, which is found in high concentrations in the placenta and fetal fluids [6,63,99]. When the uterus is invaded the uterine wall is injured causing endometritis and lesions in cotyledonal spaces. Both fetal and placental liquids are then infected, causing necrosis of the caruncle-cotyledon unions, destruction of the feto-maternal attachments and fetal death occurs due to interference in the supply of oxygen and nutrients, manifested by fetal agony. Depending on the gestational stage in which infection occurs, it can lead to abortion in the last third of gestation to term, the latter resulting in the birth of a weak animal which finally dies a few days after birth [89]. The fetus or calf does not present pathognomonic lesions, but it is common to find bronchopneumonia. The placenta is observed to be edematous, with necrotized cotyledons and inflammatory lesions [100]. Many of the infected females will not abort again; however, they can spread bacteria through their milk [20] .

8. Necropsy Findings

Necropsy findings in female buffaloes include necrotizing placentitis, ulcerative endometritis and inflammatory reactions. In the fetus, sero-hemorrhagic fluid is found in body cavities and the subepidermis, bronchopneumonia, congestion, fibrinous exudates and cellular infiltration. Fetal organs show evidence of granulomatous lesions, focal necrosis and granulomatous leptomeningitis [101-104]. In general, brucellosis does not produce clinical mastitis, and there are no apparent changes in the milk, but variations in the somatic cell count may be observed.

9. Clinical Signs

The clinical manifestations of brucellosis in domestic animals have been reviewed recently [105]. In buffalo herds where *B. abortus* is present, abortions can be seen as one of the most obvious signs, generally evident in the last third of gestation [24], however, there have also been abortions earlier in gestation [12]. In addition, there may also be infected herds with no abortions [90]. Retained placenta is common in buffaloes aborting due to brucellosis. Calves may be born normally. In males no clinical signs are common but as observed in cattle there may be epididymitis and orchitis [89]. In general, there are no systemic signs due to the action of *B. abortus* in female buffaloes, although a few buffaloes may develop transient pyrexia. Hygroma of the knee is common in affected cows but has not been described in the buffalo. It is essential to detect infected animals with proper anamnesis of the population and laboratory diagnosis.

10. Immune Response

The presence of *Brucella* spp. induces body defense mechanisms of innate immunity, such as the classical complement pathway, and action of neutrophils and macrophages. These are general resistance mechanisms for Gram negative bacteria. Neutrophils are the first cells making contact with *Brucella*. Antibodies perform opsonization of microbes, which activate the complement and facilitate phagocytosis. *Brucella* spp. can survive and multiply within the neutrophil during the course of infection, and through these cells, microbes are transported to the lymphoid tissues. To kill intracellular bacteria, it is necessary to affect the degranulation of neutrophils, with subsequent release of myeloperoxidase. Neutrophils react differently to *Brucella* spp. in different animal species, as the bacterium has more or less effective mechanisms for inhibiting degranulation and thus prevent its own destruction. Activation of the classical complement pathway can be initiated with the presence of low concentrations of IgM and IgG anti-lipopolysaccharides (LPS), thus managing bacterial lysis [106]. Macrophages conform a cell group that interacts with Brucella spp. in a particular way. The interaction between membrane receptors and LPS induces the production of interleukin-12 (IL-12), stimulating natural killer cells (NK cells) and T helper lymphocytes (LTh) CD4 +, which secrete interferon-γ (IFN-γ), favoring the development of an immune response predominantly mediated by LTh1. This subgroup of T lymphocytes primarily stimulates cellular response and are directly involved in the protection against intracellular microbes, because of its wide pattern of cytokines including IL 2, 3, 6, 12, tumor necrosis factor-α (TNF-α) and especially IFN-γ which is essential for activation of macrophages [107,108]. When microbes are phagocytized, macrophages have the ability to destroy them immediately, but as has been described for neutrophils, *Brucella* spp. is capable of inhibiting these destructive mechanisms [109]. However, the results of cytokine expression in buffaloes infected with *Brucella abortus* were inconclusive [110] and warrant further studies. The progeny of seropositive buffaloes were 6.2 times more likely to have developed serum antibodies by the time of the first calving than the progeny of seronegative buffaloes [111].

11. Sampling

All samples intended for bacterial isolation should be collected carefully and stored at 4 degrees C. The packaging used for transportation must be tight, the use of triple container to prevent human exposure is recommended. The time between sampling and arrival at the laboratory should not exceed 12 h. Samples that may be intended for organism isolation include milk, vaginal swabs, blood, fetal membranes, fetus and fetal organs. For serological diagnosis blood samples obtained by jugular or coccygeal venipuncture, collected in well-identified tubes must be submitted to the laboratory in an airtight and refrigerated container [112].

12. Prophylaxis

For prevention of brucellosis in buffaloes, the same vaccines employed for prevention of brucellosis in cattle are currently in use. Strain-19 (S19) *B. abortus* is a live vaccine applied as a single subcutaneous dose of 8-5× 10^{10} viable microorganisms [67,113,114]. S-19 is (pathogenic) a smooth strain, so antibodies generated in response to the vaccine are detected by the most conventional techniques used for diagnosis, thus the recommended application is in heifers between the ages of 3 and 8 months of age. The duration of immunity of S19 vaccine in cattle is 5-6 years. Studies comparing S19 vaccination of bubaline and bovine heifers under eight months old, demonstrated that persistence of IgG and IgM was longer in the buffalo [115]. Afzal and colleagues found that administering S19 vaccine to buffalo heifers at 6 and 12 months of age, IgG persisted longer in older animals, although no significant difference was observed when using low doses of this vaccine [113]. A reduced dose of the S19 vaccine administered by conjunctival route has been suggested for use at Brucella-infected farms [13].

A rough 45/20 killed *Brucella abortus* vaccine has been reported in the past to confer a significant degree of protection to Egyptian buffaloes against brucellosis [116] yet its further use was not documented and S19 continues to be the vaccine of choice in many countries.
Vaccine RB 51 is an attenuated, rough, mutant and stable strain derived from *B. abortus* strain 2308, which unlike S19 has no surface lipopolysaccharide O chain [116]. This vaccine has been used in several countries at different concentrations, ages and vaccination schemes with variable results, but there is a consensus that the use of this vaccine does not interfere with detection of serologically positive animals with conventional techniques [65,117-119]. The vaccine is, however, considered potentially dangerous for vaccination of adult buffalo cows as it is excreted in milk for many days subsequent to vaccination [120]. Adesiyun et al. [19] compared the pathogenicity of *B. abortus* strains isolated from cattle and buffaloes, and observed that those taken from buffaloes were less virulent in the mouse model, with no difference in histopathology. The continuity of these studies may be useful for improving prevention; especially in countries were buffalo population is increasing [19].

The use of antibiotic treatments to control infection with *Brucella* is recommended only in humans, since vaccines that are currently available are for animal use. Given the characteristics of the intracellular bacterium, antibiotic treatments are not simple and should follow the recommendations and updates to the World Health Organization (WHO) [121]. As discussed above, the use of vaccines and proper vaccination schedules is a topic of crucial importance, since vaccination is one of the pillars for the control and eradication programs for brucellosis worldwide. It is essential to use the vaccines officially approved by each country.

13. Zoonoses

Brucellosis is one of the most widespread zoonosis in the world, given that in humans there are approximately 500,000 cases per year [122]. *B. abortus* is classified in Risk Group III by the World Health Organization. The infection is considered an occupational disease in veterinarians, farmers and workers of the meat packaging industry, and is essentially acquired by the oral, respiratory or conjunctival route when handling infected animals, aborted fetuses or placentas. Technicians working in diagnostic laboratories must use personal protection and work under strict security conditions when handling infected samples or live cultures. Another way of acquiring brucellosis is through ingestion of contaminated dairy products. In countries where buffaloes live with other domestic species and interact in some way with humans, it raises the need for prevention and eradication campaigns, focused in both domestic and wild species to reduce the incidence of the disease in animals and humans [123].

In humans, the disease manifests primarily as an acute febrile illness (undulant fever) with joint and muscle pain, becoming chronic with compromised musculo-skeletal, cardiovascular and central nervous systems. It is necessary to implement public measures of awareness to decrease the incidence of brucellosis in humans, through the use of personal protective gear for workers in the meat packaging industry, veterinarians and laboratory personnel. Maneuvers performed by veterinarians in case of abortions, should be done using proper protection, especially when handling placentas and aborted fetuses, which may contain high loads of bacteria. Material recovered from abortions should be destroyed to reduce the spread of Brucella in the environment [86].
Also for families who live daily with animals potentially infected with *Brucella* spp., proper control measures should be taken during slaughter, milk ingestion and production of dairy products, and special care should be used when handling animals during calving. In countries where buffaloes are in contact with wildlife and are used for consumption, preventive measures must also be taken when handling and eating meat of other animals as these too may be infected, according to reports of many species being seropositive to *Brucella* [123,124].

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