Review Article

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Brucellosis in India: a deceptive infectious disease

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Brucellosis is an important but neglected disease in India. This zoonotic disease is present in all livestock systems and increased demand for dairy products accompanied with changing and intensified farming practices has raised the concern for increased spread and intensified transmission of this infection to the human population with increased risk of disease. Brucellosis can be controlled by mass vaccination of livestock. Human brucellosis can be treated with a combination of antibiotics but is very difficult to diagnose and requires laboratory testing for confirmation. Only a few recent studies have addressed the prevalence and importance of brucellosis as a human disease problem in India. The disease may be overlooked and misdiagnosed because of the difficult diagnosis and the absence and lack of experience with laboratory testing. Alertness of medical staff is needed to recognize and diagnose the disease. Awareness of risk groups is needed to take appropriate preventive measures and to accept control measures.

Key words Antibiotics - brucellosis - diagnosis - epidemiology - prevention - treatment

Brucellosis: Public health and economic impact

Brucellosis is one of the world's major zoonoses that still is of veterinarian, public health and economic concern in many parts of the world. Although brucellosis in livestock and transmission of infection to the human population has been significantly decreased following the instigation of effective vaccination-based control and prevention programmes in parts of the world, it remains an uncontrolled problem in regions of high endemicity such as the Mediterranean, Middle East, Africa, Latin America and parts of Asia[1,2]. Brucellosis may cause considerable economic losses. In livestock, brucellosis results in reduced productivity, abortions and weak offspring and is a major impediment for trade and export. Almost all domestic species can be affected. Human brucellosis is a severely debilitating disease that requires prolonged treatment with a combination of antibiotics often leaving permanent and disabling sequelae, and results in considerable medical expenses in addition to loss of income due to loss of working hours. Thus, its prevention, control and eradication are a major challenge for public health programmes. The presence of brucellosis in India was first established early in the previous century and since then has been reported from almost all states[3].

Veterinary aspects

Epidemiological evidence shows that in India brucellosis is present in different species of mammalian farm animals including cattle, goats, buffalo, yaks, camel, horses and pigs[3-10]. Brucellosis in cattle seems to be associated primarily with intensive farming practices in large organised dairy farms. Risk behaviours such as unrestricted trade and movement of animals, use of local cattle yards and fairs for trading, sending dry animals back to villages for maintenance, use of semen from unscreened bulls for artificial insemination and poor farm hygiene probably all contribute to the spread and transmission of the infection. Free grazing and movement with frequent mixing of flocks of sheep and goats also contribute to the high prevalence and wide distribution of brucellosis in these animals in India. Increasing demand for dairy products and protein, changing agricultural methods, and increased trade and movement of animals have caused concerns that the prevalence may increase. Therefore, there is an urgent need for the strict implementation of a control policy not only for cattle but also for small ruminants. Given the potentially huge economic and medical impact a control policy could be cost-effective[11]. India has already a policy for the control of brucellosis in dairy cattle[3].

Control and prevention of brucellosis in animals

Mass vaccination is crucial for the control and eradication of bovine, ovine and caprine brucellosis but other complementary measures that may need consideration include improved farm hygiene, restriction and control of trade and movement of animals, testing of animals and isolation and removal of infected animals. Though the existing vaccine for bovine brucellosis, the *B. abortus* strain 19 (S19), and the vaccine for ovine and caprine brucellosis, the *B. melitensis* strain Rev 1, have some undesirable traits, these have proven to be very useful under most conditions[12-15]. Given the complexity of the epidemiology of brucellosis involving various animal species, the effective control will require a long lasting and carefully controlled and monitored effort. Therefore, preventive measures will be essential to minimize the risk of infection of the human population. Such measures should include improved food hygiene including the pasteurization of milk and protection from infection of high risk groups such as milkers and other people working in the dairy industry. Health education of risk groups through community participation and health education programmes could play an important role to increase the acceptance and use of preventive measures.

Causative agent and mode of transmission

Brucellosis is caused by members of the bacterial genus *Brucella*. These are facultative intracellular Gram-negative pathogens. The ability of *Brucella* to replicate and persist in host cells is directly linked with its capacity to cause persistent disease and to circumvent innate and adaptive immunity[16,17]. The *Brucella* lipopolysaccharide (LPS), an external membrane component, has a unique structure that endows it with a very low endotoxicity, modulates the host immune response and confers resistance to antimicrobial activity and acts as virulence factor for survival and intracellular replication[18]. The bacterium may enter the body through the digestive tract, the lungs or mucosal layers and spread through the blood and the lymphatic system to any other organ where it may infect the tissue and cause localized disease. Brucellosis is almost invariably transmitted to man from infected domestic animals. Transmission from human to human, mainly mother to child, has been reported but is very rare[19]. *Brucella abortus*, *B. melitensis* and *B. suis* are pathogenic for man. Of main concern in India are *B. melitensis* and *B. abortus*. *B. melitensis* is present in and transmitted by goats and sheep and related animals and is most virulent for man. *B. abortus* is the dominant species in cattle and *B. suis* is mainly confined to pigs. In India, different *B. abortus* biotypes (types-1, 2, 4, 6 and 9) have been isolated from cattle[3]. *B. abortus* was also isolated from buffalo and from goat and sheep. *B. melitensis* biotypes 1 and 3 have been isolated from goats and sheep and cattle. *B. suis* may also be present in cattle, buffalo and goats. Though *B. melitensis* is more infectious to man than *B. abortus* and in general is the dominant causative agent of brucellosis, disease caused by infection with *B. abortus* is indistinguishable from that by *B. melitensis* and may be equally severe[20]. The

genomic sequences of *B. melitensis, B. abortus* and *B. suis* have been (partially) determined and this knowledge will help to improve our understanding of the biology and pathogenicity of these organisms and may be used to develop new acellular and safer vaccines[21-24].

Fresh milk and dairy products prepared from unpasteurized milk such as soft cheeses, yoghurts and ice creams may contain high amounts of the bacteria and consumption of these is an important cause of human brucellosis[25]. Infection also may occur through cuts and abrasions of the skin, via the conjunctiva and by inhalation. These routes of infection are important for farmers, veterinarians and butchers who all have an increased risk of infection through their contact with animals and animal products[26]. In India, historically free roaming of animals and the agrarian nature of the different traditional animal husbandry systems provided ample opportunities for intermixing of livestock through grazing at common pastures and trading at local stock yards, and hence for the transmission of the infection. The changing and fast growing dairy industry in India has resulted in intensified trade and animal movements and provide a new and increased risk in spreading the infection[3]. Human brucellosis is common in rural areas because farmers often live in close contact with their animals and consume fresh unpasteurized dairy products. However, the vending of dairy products may also bring the disease to urban areas. *Brucella* survives in the environment well. Pastures, stables and fodder may be contaminated with animal materials and secretions that contain the bacterium. Notably abortion materials may contain the bacterium in high numbers and when not properly disposed off by burying or destruction contribute to the spread of the infection. It is important to realise that open water sources such as wells can be a source of infection when contaminated with wastewater from farms. The infectious dose of in particular *B. melitensis* is very low (10 organisms for *B. melitensis*) and infective materials should be decontaminated and properly disposed of. Brucellosis in human occurs in all age groups and both males and females are affected equally in particular when dairy is the most common source of infection[27]. Brucellosis may be more common in males in areas where the disease is an occupational hazard of farmers and shepherds, butchers or veterinarians. Brucellosis in children can be very common in particular in areas with *B. melitensis*[27-29]. It is important to realise that household members of patients may have been exposed to the pathogen as well and have become infected and ill[30-32].

Human brucellosis

Clinical manifestations: Human brucellosis usually manifests as an acute or subacute febrile illness, which may persist, and progress to a chronically incapacitating disease with severe complications. The clinical picture is not specific and laboratory testing should support the diagnosis. The intermittent or remittent fever may be accompanied by malaise, anorexia and prostration. Complaints may persist for weeks or months in the absence of specific treatment. Typically, no or few objective signs are apparent that specifically point to brucellosis. Enlargement of the liver, spleen and/or lymph nodes may occur as may other signs referable to almost any other organ system. Typically these febrile patients may be referred to as patients with pyrexia of unknown origin or the symptoms and signs are confused with those of other diseases such as typhoid fever, rheumatic fever, spinal tuberculosis, pyelitis, cholecystitis, thrombophlebitis, autoimmune disease, and tumours[33-35]. Thus to an unaware physician, the diagnosis of brucellosis can be problematic. Questioning the patient at this stage about animal contacts and food habits could be helpful to raise suspicion of brucellosis when either the patient admits to own or work with livestock and mentions signs of brucellosis such as hygromas, infertility or abortions in his animals, or if the patient has a taste for fresh unpasteurized dairy. Also available epidemiological information on brucellosis as a frequent local problem in livestock should alert the physician. In all cases a blood sample should be collected from the patient and laboratory testing requested as the definite diagnosis of brucellosis is impossible without laboratory confirmation[36].

Brucellosis is acute in about half the cases, with an incubation period of two to three weeks. In the other half, the onset is insidious, developing over a period of weeks to months. Commonly patients feel better in the morning, with symptoms worsening as the day progresses. Patients have a strong desire to rest and may be depressed. Fever, chills, sweats, aches, lack of energy, joint and back pain, headache and loss of appetite are observed in majority of the patients[37]. Arthritis, constipation, abdominal pain and sleep disturbance are seen in about half of them. Cough, testicular pain/epidedymo-orchitis, rash, ill appearance, pallor, vaginal bleeding, hepatomegaly, splenomegaly, lymphadenopathy are somewhat less common. Other symptoms such as diarrhoea, jaundice, central nervous system abnormalities, cardiac murmur and pneumonia are rare. Although symptoms and signs often occur in various combinations, one study reported fever as the only sign in 44 per cent of patients with a positive blood culture for *B. melitensis* and fever with arthritis in another 42 per cent[38]. Arthritis may affect all major joints and sometimes more than one joint are affected.

Acute brucellosis may progress to a more persistent disease with localized infections or a non-specific syndrome sometimes referred to as 'chronic fatigue syndrome'[35,36]. Persistent infections often are localized to one specific organ or site[39]. During this stage *Brucella* organisms have become sequestered in cells of the reticuloendothelial system of lymph nodes, liver, spleen or bone marrow and at these sites complications may subsequently arise. Complications can be very diverse depending on the specific site of infection[40]. Osteoarticular, gastrointestinal, hepatobiliary, respiratory, genitourinary complications are observed. Bone and joint complications are the most frequent complications[41]. These include sacroiliitis, spondylitis, peripheral arthritis, osteomyelitis and bursitis. Complain of back pain with radiation down to the legs and refusal of children to walk or carry loads is indicative[42]. The liver is commonly involved in brucellosis even though liver function tests are normal or only slightly elevated. Respiratory tract complications may be seen in abattoir workers and are thought to be caused by the inhalation of bacteria[43]. Dust inhalated when cleaning stables also may cause infection. Orchitis and epididymitis are the most frequent genitourinary complications in men and may be confused with testicular cancer or tuberculosis[44]. Infection during pregnancy carries the risk of abortion or intrauterine transmission of infection to the infant[45]. Breastfeeding also may result in transmission to the breastfed infant but this probably is rare[19]. Meningitis and meningoencephalitis are the most common complications seen in neurobrucellosis[46]. The central nervous system is affected in about 5 per cent of the cases of *B. melitensis* infection and often occurs at a late stage as the main presenting manifestation. It should be noted that brucellosis may affect essentially any organ at any site and that the list of rare and unusual complications is much longer than those mentioned here. Complications of the cardiovascular system are rare but important as they have a high degree of mortality. Other examples of rare complications are those of skin and eyes[35,36].

Chronic brucellosis refers to those patients in whom symptoms persist for 12 months or more from the time of diagnosis and treatment.

Because of the deceptive nature of the clinical signs and symptoms of brucellosis the disease may be easily misdiagnosed and is often diagnosed as pyrexia of unknown origin[47]. Therefore, alertness of practitioners and health workers as well as the availability of laboratory facilities for diagnostic testing are essential.

Laboratory diagnosis: Culture from the blood of a patient provides definite proof of brucellosis[48]. Blood may be cultured using the biphasic method of Castaneda which uses both a solid and a liquid medium in the same container[49]. This method circumvents the need for subculturing and is used to limit the risk of laboratory-acquired infections. *Brucella* however, is a slow growing organism and cultures are rarely positive before the fourth day of incubation. Usually cultures become positive between the first and third week, and should be kept for at least 45 days before the culture can be concluded to be negative for *Brucella*. The modern semi-automatic culture systems have somewhat improved the speed of detection but are still too

slow to make a fast diagnosis. Another improvement which one might consider is the identification of the organism in positive blood cultures by a modification of the cold ZN staining[50]. Because of the slowness of culture one often moves to serology[51]. Most serological tests rely on the unique antigenic properties of LPS that are shared among the three *Brucella* species that cause disease in human. The use of LPS as antigen causes cross-reactivity with organisms such as *Vibrio* and *Yersinica enterocolitica* that share common features of the LPS[52]. More important in the use and interpretation of LPS based testing is the fact that in endemic areas a large proportion of the population may have developed antibodies due to previous disease or exposure.

The classical Rose Bengal test (RB) is often used as a rapid screening test[53]. RB is based on the agglutination of serum antibodies with a stained whole cell preparation of killed *Brucella*. RB is performed by mixing on a glass plate a drop of RB reagent with an equal volume of serum and agglutination is read after 2 to 4 min. The sensitivity of RB is very high (>99%) but the specificity can be disappointingly low[41,54]. As a result, the positive predictive value of the test is low and a positive test result thus requires confirmation by a more specific test. The negative predictive value of RB though is high and a negative test results excludes active brucellosis with a high degree of certainty. To increase the specificity and the positive predictive value of RB the test may be applied to a serial dilution (1:2 through 1:64) of the serum sample. The specificity of the RB increases when higher dilutions agglutinate and titres of 1:8 or 1:16 and above may be regarded as positive. This approach however inevitably results in a lower sensitivity.

For confirmation of RB the Wright or serum agglutination test (SAT) or in more sophisticated equipped laboratories enzyme linked immunosorbent assay (ELISA) may be used[52,55]. SAT is performed by mixing serial dilutions of serum, usually 1:20 through 1:2,560, with *Brucella* antigen in test tubes or in wells of an ELISA plate. After overnight incubation agglutination is read either by the unaided eye or under a binocular. As a guidance agglutination at titres of 1:160 or above is considered of diagnostic value as long as the patient has signs and symptoms of disease. In endemic areas the diagnostic threshold value will have to be set at least one titre step higher (1:320) to provide a sufficiently high specificity as many asymptomatic individuals will have titres equal to the lower threshold level of 1:160[56]. The use of the higher threshold level however severely restricts the sensitivity and clinical importance of the test. It may be important to note that none of the commercial ELISA systems has been properly evaluated using samples from culture confirmed patients with brucellosis and controls to define the sensitivity and specificity. Studies have shown that in ELISA a strong positive signal may be obtained for control samples from patients with illnesses other than brucellosis. One study suggested a specificity of only 73.4 per cent[57].

Sometimes SAT is performed in the presence of the reducing agents 2-mercaptoethanol (2-ME) or dithiothreitol (DTT). These reducing agents destroy the agglutinating activity of immunoglobulin M (IgM) leaving IgG intact[51]. The 2-ME or SAT-DTT test is used to increase the specificity of the reaction by looking at IgG only, which is important in patients with a more persistent infection.

In brucellosis, specific IgM antibodies dominate during the acute phase of the disease[58]. Specific IgG antibodies are present in the serum of patients at later stages of the illness and in the serum of relapsing patients[59]. ELISA is used to discriminate between the presence of specific IgM and IgG antibodies and to roughly access the stage of illness. SAT and the 2-ME test also are used for this purpose but are less accurate.

SAT, the 2-ME test and ELISA require special equipment and considerable expertise to perform. Not all doctors and medical facilities have easy access to a laboratory familiar with these tests. A simple and rapid diagnostic test, the *Brucella* IgM/IgG flow assay could be very useful under such conditions[58,60]. This so-called point-of care test can be performed on a drop of serum or anticoagulant treated blood

collected by finger prick and may be performed by a nurse or medical assistant. Using this test the doctor will be able to confirm the diagnosis within 15 min after examining the patient and collection of the blood sample. The sensitivity and specificity of this *Brucella* IgM/IgG flow assay for culture confirmed brucellosis is >95 per cent. Thus, by using this test the diagnosis can be made rapidly and the appropriate antibiotic can be prescribed and treatment can be started immediately. The flow assay may be used as a confirmatory test for the confirmation of RB positive samples.

Treatment: Uncomplicated acute brucellosis almost invariable responds well to appropriate antibiotic treatment[61,62]. In those patients with complications, additional treatment, including in some cases surgical intervention will be necessary. To prevent disease progression and the development of complications, treatment should start as early as possible also in patients showing signs of spontaneous improvement. In all cases it is important that the patient finishes the full course of medication because the risk of incomplete recovery and relapse is otherwise increased considerably[63]. The standard treatment of uncomplicated cases in adults and children 8 yr of age and older is 100 mg doxycycline twice a day for 6 wk plus 1 g streptomycin daily for 2 to 3 wk. Instead of streptomycin rifampicin may be given in combination with doxycycline (200 mg/day orally for 6 wk) at a dose of 600-900 mg for 6 wk. Treatment of complications such as spondylitis and osteomyelitis, neurobrucellosis and brucella endocarditis may require prolonged therapy for at least 8 wk. Other combinations such as co-trimoxazole plus doxycycline and co-trimoxazole plus rifampin have been proposed but still need further examination[64-67]. The optimal therapy for brucellosis during pregnancy has not been established[68]. Co-trimoxazole has been used successfully. Alternatively, rifampicin for at least 45 days may be used. The optimal therapy for neonates and children less than 8 yr of age remains to be established as well[68]. As for pregnant women doxycycline is contraindicated because of possible permanent staining of deciduous teeth and inhibition of bone growth[69]. Suggested therapies include trimethoprim-sulphamethoxazole (TMP/SMZ) 8/40 mg/kg/day twice daily orally for 6 wk plus streptomycin 30 mg/kg/day once daily intramuscularly for 3 wk or gentamycin 5 mg/kg/day once daily intravenously or intramuscularly for 7 to 10 days. Another alternative is TMP/SMZ plus rifampicin 15 mg/kg/day orally for 6 wk.

Human brucellosis in India

Worldwide, reported incidence of human brucellosis in endemic disease areas varies widely, from <0.01 to >200 per 100,000 population[70]. The true incidence of human brucellosis however, is unknown for most countries and no data are available for India. It is expected that the control measures that are now being instigated have become effective. It has been estimated that the true incidence may be 25 times higher than the reported incidence due to misdiagnosis and underreporting. Several publications indicate that human brucellosis can be a fairly common disease in India. A study by Mantur and coworkers[27] reported on 93 children with brucellosis who were identified by testing samples from children referred to the microbiology laboratory of the Patil Medical College in Bijapur during a period of 13 yr. The seroprevalence was 1.6 per cent by SAT (>1:160) and the diagnosis was confirmed in 43 of these paediatric patients by the isolation of *B. melitensis*. During the same period a total of 492 adult patients were diagnosed with brucellosis at the same hospital stressing the importance of childhood brucellosis. Most of the paediatric patients were shepherds and the habit of consuming fresh goat milk and the close contacts with animals were the most likely risk factors contributing to infection. Importantly, it was noted that in only 15 cases brucellosis was suspected on first diagnosis and the remaining 78 cases were initially classified as enteric fever, malaria, pyrexia of unknown origin, and rheumatic arthritis. Fever and or joint pain notably of the knee and hip were the only clinical manifestations in the majority of the patients. It was concluded that alertness of clinicians and close collaboration with the microbiologist are essential even in endemic areas to correctly diagnose and treat brucellosis. Other studies reported cases of pancytopaenia, neurobrucellosis, pulmonary brucellosis, spondylitis and sacroiliitis, and

(poly)arthritis and it is important to consider brucellosis in the differential diagnosis of these and other unusual presentations[71-76].

Since many patients with brucellosis present with fever as the only manifestation, other groups have investigated the prevalence of brucellosis in patients diagnosed with fever of unknown origin (FUO). Handa and coworkers identified 4 cases with acute brucellosis in a group of 121 patients with FUO[77]. Sen and coworkers identified 28 (6.8%) seropositive cases in a group of 414 patients with FUO and Kadri and coworkers identified 28 (0.8%) seropositive cases in a group of 3,532 patients with FUO[78,79]. The seroprevalence in specific risk groups such as abattoir workers is much higher[76,80].

Reporting, control and prevention

No vaccine is available for the prevention of brucellosis in human. Reporting of brucellosis to health authorities is extremely important and knowledge of the disease prevalence can be used to prioritize a disease control policy for brucellosis and to alert health staff. Brucellosis should be controlled by vaccination of livestock. Mass vaccination of livestock together with other measures such as movement control and testing and isolation of infected animals can effectively control brucellosis in the animal population and eventually reduce the transmission to the human population. India already has developed a plan for the control of bovine brucellosis[3]. However, as brucellosis transmitted from small ruminants poses a significant health risk further efforts will be needed to control brucellosis in goats and sheep as well. While effective control measures still need to be implemented, doctors and other health workers may help patients and risk groups to prevent brucellosis by teaching them essential methods to prevent exposure to the pathogen such as boiling of milk and to avoid the consumption of dairy products prepared from unpasteurized milk. Farmers, shepherds, milkers, veterinarians and other workers in the dairy industry should know about the measures required to prevent the risk of exposure and infection. They also should know that vaccination of their animals for brucellosis is important. Vaccination not only is important to improve the health of their animals but also is an important step to reduce the risk of severe illness and disability for themselves and their family members. Vaccination of livestock is relatively cheap and will increase the value and productivity of their animals.

References

1. Corbel MJ. Brucellosis: an overview. *Emerg Infect Dis* 1997; *3* : 213-21.

2. Refai M. Incidence and control of brucellosis in the Near East region. *Vet Microbiol* 2002; *90* : 81-110.

3. Renukaradhya GJ, Isloor S, Rajasekhar M. Epidemiology, zoonotic aspects, vaccination and control/eradication of brucellosis in India. *Vet Microbiol* 2002; *90* : 183-95.

4. Isloor S, Renukaradhya GJ, Rajasekhar M. A serological survey of bovine brucellosis in India. *Rev Sci Tech* 1998; *17* : 781-5.

5. Mehra KN, Dhanesar NS, Chaturvedi VK. Sero-prevalence of brucellosis in bovine in Madhya Pradesh. *Indian Vet J* 2000; *77* : 571-3.

6. Chakraborty M, Patgiri GP, Barman NN. Application of delayed-type hypersensitivity test (DTH) for the diagnosis of bovine brucellosis. *Indian Vet J* 2000; *77* : 924-5.

7. Desai T, Krishnappa G, Upadhye AS. Incidence of brucellosis in sheep, goats and some human risk groups. *Mysore J Agri Sci* 1995; *29* : 348-51.

8. Kumar P, Singh DK, Barbuddhe SB. Serological evidence of brucellosis in sheep and goats. *Indian J Anim Sci* 1997; *67* : 180-2.

9. Mrunalini N, Rama SP, Pandarinath GN, Ramakrishna RM. Control of brucellosis in goats in a farm. *Indian Vet J* 2000; *77* : 932-5.

10. Singh SV, Agarwal GS, Batra HV, Gupta VK, Singh N. Monitoring of *Brucella* infection associated with reproductive losses using multiple serological tests in organized goat and sheep flocks. *Indian J Anim Sci* 2000; *70* : 154-6.

11. Roth F, Zinsstag J, Orkhon D, Chimed-Ochir G, Hutton G, Cosivi O, *et al*. Human health benefits from livestock vaccination for brucellosis: case study. *Bull World Health Organ* 2003; *81* : 867-76.

12. Moriyon I, Grillo MJ, Monreal D, Gonzalez D, Marin C, Lopez-Goni I, *et al*. Rough vaccines in animal brucellosis: structural and genetic basis and present status. *Vet Res* 2004; *35* : 1-38.

13. Minas A, Minas M, Stournara A, Tselepidis S. The "effects" of Rev-1 vaccination of sheep and goats on human brucellosis in Greece. *Prev Vet Med* 2004; *64* : 41-7.

14. Schuurman HJ. The serological response of adult cattle to vaccination with reduced dose *Brucella abortus* S19, a trial under Zambian conditions. *Vet Q* 1983; *5* : 94-6.

15. Schurig GG, Sriranganathan N, Corbel MJ. Brucellosis vaccines: past, present and future. *Vet Microbiol* 2002; *90* : 479-96.

16. Gorvel JP, Moreno E. Brucella intracellular life: from invasion to intracellular replication. *Vet Microbiol* 2002; *90* : 281-97.

17. Ficht TA. Intracellular survival of Brucella: defining the link with persistence. *Vet Microbiol* 2003; *92* : 213-23.

18. Lapaque N, Moriyon I, Moreno E, Gorvel JP. Brucella lipopolysaccharide acts as a virulence factor. *Curr Opin Microbiol* 2005; *8* : 60-6.

19. Palanduz A, Palanduz S, Guler K, Guler N. Brucellosis in a mother and her young infant: probable transmission by breast milk. *Int J Infect Dis* 2000; *4* : 55-6.

20. Dokuzoguz B, Ergonul O, Baykam N, Esener H, Kilic S, Celikbas A, *et al*. Characteristics of *B. melitensis* versus *B. abortus* bacteraemias. *J Infect* 2005; *50* : 41-5.

21. DelVecchio VG, Kapatral V, Elzer P, Patra G, Mujer CV. The genome of *Brucella melitensis*. *Vet Microbiol* 2002; *90* : 587-92.

22. DelVecchio VG, Kapatral V, Redkar RJ, Patra G, Mujer C, Los T, *et al*. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. *Proc Natl Acad Sci USA* 2002; *99* : 443-8.

23. Paulsen IT, Seshadri R, Nelson KE, Eisen JA, Heidelberg JF, Read TD, *et al*. The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. *Proc Natl Acad Sci USA* 2002; *99* : 13148-53.

24. Sanchez DO, Zandomeni RO, Cravero S, Verdun RE, Pierrou E, Faccio P, *et al*. Gene discovery through genomic sequencing of *Brucella abortus*. *Infect Immun* 2001; *69* : 865-8.

25. Bikas C, Jelastopulu E, Leotsinidis M, Kondakis X. Epidemiology of human brucellosis in a rural area of north-western Peloponnese in Greece. *Eur J Epidemiol* 2003; *18* : 267-74.

26. Omer MK, Assefaw T, Skjerve E, Tekleghiorghis T, Woldehiwet Z. Prevalence of antibodies to *Brucella* spp. and risk factors related to high-risk occupational groups in Eritrea. *Epidemiol Infect* 2002; *129* : 85-91.

27. Mantur BG, Akki AS, Mangalgi SS, Patil SV, Gobbur RH, Peerapur BV. Childhood brucellosis - a microbiological, epidemiological and clinical study. *J Trop Pediatr* 2004; *50* : 153-7.

28. Ciftci E, Ince E, Dogru U. Pyrexia of unknown origin in children: a review of 102 patients from Turkey. *Ann Trop Paediatr* 2003; *23* : 259-63.

29. Caksen H, Arslan S, Oner AF, Cesur Y, Ceylan A, Atas B, *et al*. Childhood brucellosis is still a severe problem in the eastern region of Turkey. *Trop Doct* 2002; *32* : 91-2.

30. Gotuzzo E, Seas C, Guerra JG, Carrillo C, Bocanegra TS, Calvo A, *et al*. Brucellar arthritis: a study of 39 Peruvian families. *Ann Rheum Dis* 1987; *46* : 506-9.

31. Almuneef MA, Memish ZA, Balkhy HH, Alotaibi B, Algoda S, Abbas M, *et al*. Importance of screening household members of acute brucellosis cases in endemic areas. *Epidemiol Infect* 2004; *132* : 533-40.

32. Dhamdhere MR, Bhagwat RB, Sainani GS, Deshmukh PY, Patil SD, Gagrani LJ. Outbreak of brucellosis in a family. *Indian J Med Sci* 1964; *18* : 145-9.

33. Lulu AR, Araj GF, Khateeb MI, Mustafa MY, Yusuf AR, Fenech FF. Human brucellosis in Kuwait: a prospective study of 400 cases. *Q J Med* 1988; *66* : 39-54.

34. Madkour MM. Epidemiologic aspects. In: Madkour MM, editor. *Madkour's brucellosis*. New York: Springer; 2001 p. 21-32.

35. Young EJ. *Brucellosis: clinical and laboratory aspects*. In: Corbel MJ, editor. Florida, USA: CRC Press Inc; 1989.

36. Young EJ. An overview of human brucellosis. *Clin Infect Dis* 1995; *21* : 283-9.

37. Gur A, Geyik MF, Dikici B, Nas K, Cevik R, Sarac J, *et al*. Complications of brucellosis in different age groups: a study of 283 cases in southeastern Anatolia of Turkey. *Yonsei Med J* 2003; *44* : 33-44.

38. Memish Z, Mah MW, Al Mahmoud S, Al Shaalan M, Khan MY. Brucella bacteraemia: clinical and laboratory observations in 160 patients. *J Infect* 2000; *40* : 59-63.

39. Ficht TA. Intracellular survival of *Brucella*: defining the link with persistence. *Vet Microbiol* 2003; *92* : 213-23.

40. Hasanjani Roushan MR, Mohrez M, Smailnejad Gangi SM, Soleimani-Amiri MJ, Hajiahmadi M. Epidemiological features and clinical manifestations in 469 adult patients with brucellosis in Babol, Northern Iran. *Epidemiol Infect* 2004; *132* : 1109-14.

41. Barroso-Garcia P, Rodriguez-Contreras Pelayo R, Gil-Extremera B, Maldonado-Martin A, Guijarro-Huertas G, Martin Salguero A, *et al*. Study of 1,595 brucellosis cases in the Almeria province (1972-1998) based on epidemiological data from disease reporting. *Rev Clin Esp* 2002; *202* : 577-82.

42. Gokhale YA, Bichile LS, Gogate A, Tillu AV, Zamre A. Brucella spondylitis: an important treatable cause of low backache. *J Assoc Physicians India* 1999; *47* : 384-8.

43. Karimi A, Alborzi A, Rasooli M, Kadivar MR, Nateghian AR. Prevalence of antibody to *Brucella* species in butchers, slaughterers and others. *East Mediterr Health J* 2003; *9* : 178-84.

44. Kocak I, Dundar M, Culhaci N, Unsal A. Relapse of brucellosis simulating testis tumor. *Int J Urol* 2004; *11* : 683-5.

45. Giannacopoulos I, Eliopoulou MI, Ziambaras T, Papanastasiou DA. Transplacentally transmitted congenital brucellosis due to *Brucella abortus*. *J Infect* 2002; *45* : 209-10.

46. Kochar DK, Kumawat BL, Agarwal N, Shubharakaran N, Aseri S, Sharma BV, *et al*. Meningoencephalitis in brucellosis.*Neurol India* 2000; *48* : 170-3.

47. Ergonul O, Willke A, Azap A, Tekeli E. Revised definition of 'fever of unknown origin': limitations and opportunities. *J Infect* 2005; *50* : 1-5.

48. Al Dahouk S, Tomaso H, Nockler K, Neubauer H, Frangoulidis D. Laboratory-based diagnosis of brucellosis - a review of the literature. Part I: Techniques for direct detection and identification of *Brucella* spp. *Clin Lab* 2003; *49* : 487-505.

49. Mantur BG, Mangalgi SS. Evaluation of conventional castaneda and lysis centrifugation blood culture techniques for diagnosis of human brucellosis. *J Clin Microbiol* 2004; *42* : 4327-8.

50. Joshi PA, Kulkarni RD, Powar RM. Modified cold Z-N staining for presumptive identification of Brucella. *Indian J Med Res* 2005; *121* : 108-10.

51. Al Dahouk S, Tomaso H, Nockler K, Neubauer H, Frangoulidis D. Laboratory-based diagnosis of brucellosis - a review of the literature. Part II: serological tests for brucellosis. *Clin Lab* 2003; *49* : 577-89.

52. Munoz PM, Marin CM, Morreal D, Gonzales D, Garin-Bastuji B, Diaz R, *et al*. Efficacy of several serological tests and antigens for diagnosis of bovine brucellosis in the presence of false-positive serological results due to *Yersinia enterocolitica* O:9. *Clin Diag Lab Immunol* 2005; *12* : 141-51.

53. Ruiz-Mesa JD, Sanchez-Gonzalez J, Reguera JM, Martin L, Lopez-Palmero S, Colmenero JD. Rose Bengal test: diagnostic yield and use for the rapid diagnosis of human brucellosis in emergency departments in endemic areas. *Clin Microbiol Infect* 2005; *11* : 221-5.

54. Kiel FW, Khan MY. Analysis of 506 consecutive positive serologic tests for brucellosis in Saudi Arabia. *J Clin Microbiol* 1987; *25* : 1384-7.

55. Mathai E, Singhal A, Verghese S, D'Lima D, Mathai D, Ganesh A, *et al*. Evaluation of an ELISA for the diagnosis of brucellosis. *Indian J Med Res* 1996; *103* : 323-4.

56. Martin Moreno S, Guinea Esquerdo L, Carrero Gonzalez P, Visedo Orden R, Garcia Carbajosa S, Calvo del Olmo T, *et al*. Diagnosis of brucellosis in an endemic area. Evaluation of routine diagnostic tests. *Med Clin (Barc)* 1992; *98* : 481-5.

57. Gokhale YA, Ambardekar AG, Bhasin A, Patil M, Tillu A, Kamath J. Brucella spondylitis and sacroiliitis in the general population in Mumbai. *J Assoc Physicians India* 2003; *51* : 659-66.

58. Smits HL, Abdoel TH, Solera J, Clavijo E, Diaz R. Immunochromatographic *Brucella*-specific immunoglobulin M and G lateral flow assays for rapid serodiagnosis of human brucellosis. *Clin Diagn Lab Immunol* 2003; *10* : 1141-6.

59. Ariza J, Pellicer T, Pallares R, Foz A, Gudiol F. Specific antibody profile in human brucellosis. *Clin Infect Dis* 1992; *14* : 131-40.

60. Irmak H, Buzgan T, Evirgen O, Akdeniz H, Demiroz AP, Abdoel TH, *et al*. Use of the *Brucella* IgM and IgG flow assays in the serodiagnosis of human brucellosis in an area endemic for brucellosis. *Am J Trop Med Hyg* 2004; *70* : 688-94.

61. Solera J, Martinez-Alfaro E, Espinosa A. Recognition and optimum treatment of brucellosis. *Drugs* 1997; *53* : 245-56.

62. Solera J. Treatment of human brucellosis. *J Med Liban* 2000; *48* : 255-63.

63. Solera J, Martinez-Alfaro E, Espinosa A, Castillejos ML, Geijo P, Rodriguez-Zapata M. Multivariate model for predicting relapse in human brucellosis. *J Infect* 1998; *36* : 85-92.

64. Roushan MR, Gangi SM, Ahmadi SA. Comparison of the efficacy of two months of treatment with co-trimoxazole plus doxycycline vs. co-trimoxazole plus rifampin in brucellosis. *Swiss Med Wkly* 2004; *134* : 564-8.

65. Solera J, Geijo P, Largo J, Rodriguez-Zapata M, Gijon J, Martinez-Alfaro E, *et al*. A randomized, double-blind study to assess the optimal duration of doxycycline treatment for human brucellosis. *Clin Infect Dis* 2004; *39* : 1776-82.

66. El Miedany YM, El Gaafary M, Baddour M, Ahmed I. Human brucellosis: do we need to revise our therapeutic policy? *J Rheumatol* 2003; *30* : 2666-72.

67. Bayindir Y, Sonmez E, Aladag A, Buyukberber N. Comparison of five antimicrobial regimens for the treatment of brucellar spondylitis: a prospective, randomized study. *J Chemother* 2003; *15* : 466-71.

68. Young EJ. *Brucella* species (Brucellosis). In: Yu VL, Weber R, Raoult D, editors. *Antimicrobial, therapy and vaccines*. Vol. I: Microbes. New York, USA: Apple Tree Productions, LLC; 2003 p. 121-40.

69. Lochary ME, Lockart PB, Williams WT. Doxycicline and staining of permanent teeth. *Pediatr Infect Dis J* 1998; *17* : 429-31.

70. Boschirali ML, Foulongue V, Callaghan D. Brucellosis: a worldwide zoonosis. *Curr Opin Microbiol* 2001; *4* : 658-64.

71. Banerjee TK, Pal AK, Das S. Neurobrucellosis presenting as acute meningoencephalitis. *Neurol India* 1999; *47* : 160.

72. Kochar DK, Agarwal N, Jain N, Sharma BV, Rastogi A, Meena CB. Clinical profile of neurobrucellosis - a report on 12 cases from Bikaner (north-west India). *J Assoc Physicians India* 2000; *48* : 376-80.

73. Kalla A, Chadda VS, Gauri LA, Gupta A, Jain S, Gupta BK, *et al*. Outbreak of polyarthritis with pyrexia in Western Rajasthan. *J Assoc Physicians India* 2001; *49* : 963-5.

74. Kadri MS, Tanvir M, Chowdhary ND, Hassan G, Gaash B. Pancytopenia in a brucellosis case investigated for pyrexia of unknown origin. *JK Practitioner* 2003; *10* : 222-3.

75. Kochar DK, Sharma BV, Gupta S, Jain R, Gauri LA, Srivastava T. Pulmonary manifestations in brucellosis: a report on seven cases from Bikaner (north-west India). *J Assoc Physicians India* 2003; *51* : 33-6.

76. Chadda VS, Soni PK, Gupta A, Gupta BK, Chadda S, Nayak KC. Incidence of brucellosis in arthritis and chronic low back pain in high risk group. *J Assoc Physicians India* 2004; *52* : 338.

77. Handa R, Singh S, Singh N, Wali JP. Brucellosis in north India: results of a prospective study. *J Commun Dis* 1998; *30* : 85-7.

78. Sen MR, Shukla BN, Goyal RK. Seroprevalence of brucellosis in and around Varanasi. *J Commun Dis* 2002; *34* : 226-7.

79. Kadri SM, Rukhsana A, Laharwal MA, Tanvir M. Seroprevalence of brucellosis in Kashmir (India) among patients with pyrexia of unknown origin. *J Indian Med Assoc* 2000; *98* : 170-1.

80. Barbuddhe SB, Kumar P, Malika SV, Singh DK, Gupta LK. Seropositivity for intracellular bacterial infections among abattoir associated personnels. *J Commun Dis* 2000; *32* : 295-9.

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