EFFECTIVE METHODS FOR APPROPRIATE DIAGNOSIS OF BRUCELLOSIS IN HUMANS AND ANIMALS (REVIEW ARTICLE)

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ABSTRACT: Brucellosis is one of the most common diseases among human that identification and control of disease transmission methods can promote public health. Clinical signs alone are not sufficient for brucellosis diagnosis. Hence, a sensitive, specific, rapid and inexpensive method is required. Early and appropriate diagnosis of this disease is effective in improving public health as well as disease control and eradication. Several serological tests for probable diagnosis of Brucella infection were used in evaluation of antibodies against Brucella. Using new methods such as Elisa has higher sensitivity and specificity than standard SAT test and complement fixation which can show both G and M immunoglobulins. It is also suitable for examining certain class of immunoglobulin. Research and studies have shown that ELISA is a complete method for in vitro detection of chronic disease, especially when other tests results are negative. In addition to this method, all unique and specific immunoglobulin in tested serum appear with high speed and accuracy. Another diagnostic method is PCR, which has higher sensitivity and specificity in comparison with serologic methods for diagnosis of human brucellosis. PCR shows similar sensitivity as 16srRNA using L7/L12 gene. It can be used in diagnosis of human brucellosis. Another diagnostic method is identification of different forms of IL-10 gene, which is a cytokine. It inactivates macrophages and infects the susceptible subject with brucellosis. Therefore, identification of different forms of IL-10 gene is considered as effective method for diagnosis of the disease. It’s recommended to use this new and effective method because many of these methods can overcome limitations of traditional methods.

Keywords: Brucellosis, Diagnosis, Human, Animal

INTRODUCTION

Malta fever or brucellosis is commonly called contagious abortion in animals. This is one of the infectious diseases transmissible between humans and animals. This is also called undulant fever, frenzy fever and Mediterranean fever. This disease occurs in all seasons; however, it is more common in springs and falls during calving and lactation periods in animals. This disease was first discovered by David Bruce in 1887 from spleen of English soldiers killed in war in Malta Island. Therefore, it is called brucellosis disease (AGHA, 2002). Brucellosis is still common in many countries in Mediterranean domain, Middle East, Arabian Peninsula, Central and South America, Asia and Africa. Only 17 countries such as Norway, Scotland, Switzerland, United Kingdom, Denmark, Romania and Netherlands, Manish Island, Sweden and several other countries were formally declared free of Brucellosis. In some countries, like the United States, this disease is primarily considered as an occupational hazard (Gotuzzo et al., 1998). However, this bacterial infection is not restricted to specific jobs in other countries such as Iran. In addition, it is one of the most important disease common between animals and humans. This is also one of the most important health problems. Prevalence of Malta fever in humans and animals depend directly on prevalence of brucellosis. Therefore, this disease should be inevitably controlled and eradicated in order to avoid economic losses and health risks caused by this disease (Meglid et al., 2010). Pathogen of this disease is a gram-negative coccobacillus Brucella, which is a small, aerobic, non-motile and non-sporic and non-capsulic. These bacteria grow slowly. Although Brucella grow properly at 37°C and PH = 6.7 in Brucella broth medium, Brucella colonies usually grow in solid media as smooth, clear, bluish to white and amber colonies. Brucella Canis and Brucella Ovis grow as rough colonieand sometimes as mucoid colonies. There are a few numbers of strains of bacteria with lipopolysaccharide in their outer membranes, which are less virulent. Brucella Abortus and Brucella Ovis need a medium containing 5% to 10% carbon dioxide in initial isolation.

Brucella’s resistance in various conditions: Survival of Brucella species depends on type of nutrients, amount of heat and moisture and PH-level. The pathogen survives in proper environmental conditions with appropriate humidity, in animal feces and urine for weeks and sometimes months. Brucella species can survive in frozen meat.
for three weeks, in raw milk for 10 days, in a fresh cheese up to three months and in ice cream and cream for a month. These bacteria are not destroyed with freezing. These bacteria cannot survive in yogurt due to presence of lactic acid. This organism survives for 40 days in dry soil contaminated with urine, feces and discharge and products of infected pregnant animals. This bacterium is much more resistant in moist soil. This survives in animal feces in open air for 100 days while it survives at 8°C more than one year. However, it is destroyed at 60°C in 10 minutes. Nevertheless, the number of these organisms severely decreases within a few days by smoking, salting and freezing the infected meat (AGHA, 2002).

**Brucella types:** there are four types of Brucella detected as agent of majority of brucellosis infection in humans.

- **Brucella Melitensis** has three serotypes. Most cases are infected by direct or indirect contact with sheep and goats while little number of cases occurs due to contact with camels and cattle. Brucella melitensis is a major cause of brucellosis in humans. Serotype 1 is common in Iran.
- **Brucella Abortus** has seven serotypes. Most cases occur due to contact with cattle while few cases occur due to contact with camels and yak. This type of Brucella is less virulent than Brucella melitensis in humans. Serotype 3 is common in Iran.
- **Brucella Suis** has five serotypes. The infected cases occur due to contact with pigs. This type of Brucella can cause abortion in pigs. Serotypes 1 and 3 cause infection in humans.
- **Brucella Canis** occurs due to contact with dogs. It causes asymptomatic infection in humans.
- **Brucella Ovis**, Brucella Maris, Brucella neotobut are less common that above-mentioned four strains (Cecil, 2000; Oxford, 1996).
- **Brucella melitensis** is the most common pathogen of this disease in humans. Any disease caused by Brucella Canis and Brucella Maris is extremely rare in humans. Brucella Ovis causes testicular swelling in ram while it does not cause disease in humans. It is not proved whether Brucella neotome and Brucella microti (pathogen of brucellosis in rodents) cause disease in humans.

**Brucellosis transmission methods in humans and animals**

Malta fever is transmitted to animals by mating, consumption of infected milk, respiratory transmission in folds and stalls, contact with uterine secretions of infected cattle or infected and aborted fetuses and placenta. This infection is mostly transmitted to animals through ingestion of food contaminated with fecal contents. Due to acidic vaginal medium of animals, the bacteria that enter into vagina through sex may not be pathogenic.

Brucella bacteria enter human body through several ways. It infects human body orally through consumption of infected unpasteurized milk and dairy products, which is one of the most common methods of transmitting the disease. Other consumers products made from infected animals such as liver, meat, blood consumed raw or undercooked are also considered as source of infection. Transmission of infection through inhalation routes is usually considered as an occupational hazard among shepherds, animal transporters, farm workers, slaughterhouse workers, veterinarians and veterinary technicians. Respiration is the most common way of transmitting the infection among laboratory workers. The bacteria may randomly infect cases during butchering. Veterinarians or animal husbandry workers may be infected through skin contact with infected secretions of livestock. Infection of eyes with infected fecal material during animal care is also common. Accidental injection of live Brucella vaccine (Rev1, S19 and RB51) to animals may lead to mild form of the disease. No case of infection transmission from human to human was reported. However, the risk of transmission through intrauterine, breastfeeding, blood transfusions, bone marrow transplants and sexual contact still exist (Megid et al., 2010).

**Brucellosis diagnosis in humans and animals**

Risk of the disease and observing clinical symptom are evident in case of contact with bacteria. However, the disease can be diagnosed in in vitro observation. Brucellosis disease can be diagnosed early in livestock through blood serum, milk, aborted fetuses in morbidly appropriate samples sent to the laboratory using screening tests such as Rose Bengal RBPT, Milk Ring Test (MRT) and direct ELISA (D-ELISA). Subsequently, the disease is confirmed and diagnosed by complementary tests such as Wright seroagglutination test (SAT), 2ME Mercaptoethanol test, indirect ELISA (I-ELISA) and competitive ELISA (C-ELISA). Cultures of clinical specimens and isolation of infectious pathogens indicate definite diagnosis of the disease.

Brucellosis laboratory tests in humans are often performed in case the patients visit hospitals. Brucellosis is diagnosed based on epidemiologic history, clinical findings and high or increasing Brucella antibody titers with or without positive cultures of blood or other fluids and tissues. Many different studies were conducted in order to achieve faster and better diagnostic procedures (Megid et al., 2010; Kokoglu et al., 2006). The Golden Standard for diagnosis of this disease through isolation of bacteria from blood, bone marrow or septic aggregation is not reliable. However, in practice, obtaining a positive blood culture and using brucellosis diagnostic methods are associated with several problems such as time consuming, risk of personnel infection and getting false negative results. Therefore, serological testing is essential (Haddadi et al., 2006; Malik, 1997; Elbeltagy, 2001; Alvarez et al., 2000).

**Diagnosis through clinical symptoms**

**- Symptoms in humans:** Disease latency (From time of contact with source of infection to occurrence of symptoms) is often between 1 and 3 weeks. However, it is sometimes up to 6 months. Based on severity of the disease, symptoms are manifested in three forms: acute, sub-acute and chronic.
The acute form: the patient suffers from sudden chills, general body aches, especially back pain and intense sweating. He may lose his appetite and suffer from weaknesses and lassitude. In addition, symptoms may occur no more than three months passed from infection with the disease.

Sub-acute form: it begins silently. The patient mainly complains of weakness and fatigue. The symptoms may manifest from 3 to 12 months since beginning of the disease.

Chronic form: chronic form of this disease may occur in case that more than one year has passed from time of diagnosis when the patient is still suffering from the disease. The subjects who may show those symptoms such as fever, lack of appetite, muscle aches, and night sweating or have a history of contact with infected animals, are suspected of having brucellosis. Those who consume infected dairy products should be evaluated in terms of brucellosis. This disease is differentially diagnosed due to variety of clinical symptoms in accordance with many other infectious and non-infectious diseases (Megid et al., 2010).

Symptoms in animals
Brucellosis highly reproduces in milk glands and uterus of pregnant and lactating animals. These organisms reside in chorionic epithelial cells, which cause necrosis in placental cotyledons. This usually leads to abortion of fetuses in pregnant animals due to intrauterine infection. Animals usually recover on their own. However, the infected animals dispose pathogenic organisms through their uterine, urine and milk secretions for a variable period and cause infection in other animals or humans. On the other hand, cattle and goats may remain infected during their entire life. In addition, animals with chronic brucellosis may not suffer from abortion. It is not likely that they produce less milk. Moreover, they can transmit the disease to other animals. They may be an important source of human infection through their milk products. Occurrence of clinical signs of disease in livestock is dependent on level of safety of the herds. In non-vaccinated flocks, abortion is the most important symptom of the disease. Abortion occurs in cattle after the fifth month of pregnancy while it occurs in the last two months of pregnancy in sheep and goats. Although incidence of abortion is observed in the second and ongoing months of pregnancy in a number of infected animals, most animals do not miscarriage in the second pregnancy and afterwards. Retained placenta, metritis, arakit, hygroma, decreased milk production, permanent or temporary infertility, delay in reproductive seasons and increased lactation intervals can be cited as other symptoms of this disease. Those symptoms such as fever, respiratory impairment, weight loss, diarrhea and limping may be observed in acute form of the disease (Megid et al., 2010).

Evaluation of clinical signs and symptom in various studies: In a study conducted in Turkey, 78.3% had fever while 77.5% had arthralgia, 72.5% showed sweating and 7.5% had epididymoorchitis. In a study carried out in Saudi Arabia, 79.2% had fever while 70.4% had arthralgia and 3.8% had splenomegaly (Kokoglu et al., 2006; Fallatah et al., 2005).

The most common symptoms were fever (67.22%), sweating (73.25%), malaise and fatigue (65.45%), arthralgia (29.15%), headache and coughing (Haddadi et al., 2006).

In another study conducted on 104 patients in Saudi Arabia, 100% had fever while 96.2% had sweating, 70% had headache, 73.1% had back pain and 76.9% had arthralgia (Malik, 1997). Hasanjani Roushan et al. conducted a study on 404 subjects and showed that the most common symptoms were fever, sweating and arthralgia (Hasanjani Roushan et al., 2004). In a study conducted in Kuwait, the most common symptoms were sweating, fever, headache and arthralgia with respectively 91%, 40% 49% and 23% incidences (Mousa et al., 1987). In another study carried out in Saudi Arabia, 100% had fever while 46.2% had hepatomegaly, 42.3 had splenomegaly and 26.6% had arthritis (Fallatah et al., 2005).

In another study conducted in Turkey, 66.6% had fever, 63.3% had hepatomegaly, 56.6% had splenomegaly, 23.3% had arthritis and 6.8% had epididymo-orchitis (Namiduru et al., 2003). In a study conducted in Kuwait, 27% had hepatomegaly while 37% had arthritis (Mousa et al., 1988). Rasoulinejad et al. conducted a study on 505 patients and showed that 42% had hepatomegaly while 34% had splenomegaly and few cases had arthritis. The most involved joints were knees, hips and sacroiliac joints (Rasoulinejad et al., 2002). In another study conducted in Iran, the most involved joints were knee, sacroiliac and spondylitis joints (Hasanjani Roushan et al., 2004). In a survey conducted on 238 patients in Turkey over 6 years, 36.5% patients had osteoarticular involvement. The most involved joints were respectively sacroiliac, peripheral arthritis, spondylitis and bursitis (Tasova et al., 1999) in two studies conducted in Spain; the most involved joints were sacroiliac and spondylitis (Ariza et al., 1993; Gonzalez et al., 1999).

Laboratory
Cell blood count: monocytosis, lymphocytosis and anemia were observed in a study conducted in Turkey (Tasova et al., 1999). In the study conducted by Roshan et al, 84.5% had normal WBC while 80.8% had normal HB and 80.7% had normal ESR and 60.4% had normal CRP (Hasanjani Roushan et al., 2004).

Serologic Tests: Several serological tests were used for probable diagnosis of Brucella infection in evaluation of antibodies against Brucella. Tube standard agglutination test or Wright Test is cited as one of the oldest test invented by Wright and Smith in 1897 (Tohme et al., 2001). Coombs Wright Methods (Anti-human globulin test), complement fixation (CFT), agglutination 2 - mercaptoethanol (2-ME), Ring Rose Bengal test and fast agglutination on lam are cited as other serological valuable methods used for diagnosis of brucellosis (Ariza et al., 1992; Gazapo et al., 1989; Lulu et al., 1986; Peraza et al., 2004). Nowadays, countries such as Germany, Cuba, the United States and China proceeded to produce Elisa kits to detect immune response against brucellosis in humans and or

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animals. According to research conducted on many industrial Elisa kits, human immunoglobulin antibodies such as IgM or IgG were solely used (Ferreira et al., 2003; Hajia 2006). In a number of these kits, IgM and IgG were used mutually as conjugate. In this case, the kits could detect both acute and chronic phases of the disease (Ferreira et al., 2003; Hajia 2006; Ertek et al., 2006; Henk et al., 2003).

In a study conducted by Araj et al. they not only compared tube standard agglutination test (SAT) with ELISA test, but also introduced ELISA test as the selected test for diagnosis in patients clinically suspected of having brucellosis (Araj et al., 2005). In a study conducted in Kuwait on application of ELISA for diagnosis of Brucella, sensitivity and specificity of IgG ELISA was detected as 98% for patients with acute or chronic brucellosis. The researchers also expressed that ELISA test is a rapid, sensitive and specific method for diagnosis of Brucella bacteria in humans provided that a view of immunoglobulin classes be prepared in diagnosis of acute and chronic brucellosis. Therefore, ELISA method can be described as a selected method for serological diagnosis of this disease (Ferreira et al., 2003). Chaudhuri et al. used a recombinant protein with 28 kilo dalton weight in outer membrane (OMP28) of Brucella melitensis as antigen. This antigen can induce immune system in cattle, sheep, goats and dogs. The sensitivity and specificity caused by this recombinant antigen were respectively equal to 88.7% and 93%, which were less than sensitivity and specificity compared to SLPS antigen used in this study (Chaudhur et al., 2010).

A study was conducted in Iran using ELISA method with kit designed to diagnose Brucellosis in human and livestock on 40 serum samples (10 human serum and 30 livestock samples). It was reported that Wright test was positive. It showed that all samples with designed ELISA kits showed positive test results. In addition, 86 samples showed negative results among 89 negative serum samples (41 human serum samples and 48 livestock serum samples). In this study, sensitivity was obtained as 95.83% for livestock kits while sensitivity was obtained as 100% and specificity as 97.56% for human kits and sensitivity was obtained as 100 % and specificity as 96.73% for combined kits. Accordingly, the threshold or (Cut off) was also determined as 0.13. These researchers showed that this kit could simultaneously diagnose brucellosis in animals or humans using two conjugates. High accuracy, sensitivity and specificity with fast testing procedure are cited as advantages of this method compared with other serological tests. Using a very small amount of patient’s serum compared to other indices also resulted in high speed, high accuracy, ease of testing, high sensitivity, diagnosis of the disease in both acute and chronic phases and reducing the time for brucellosis diagnosis to 75 minutes from 24 hours with tube standard agglutination test (SAT) (Samavati et al., 2012).

Type-1 immunity is important in controlling Brucella and macrophages infection. Interleukin-10 is a type 2 cytokine, which deactivates macrophages and has adverse effects on the disease (Serre et al., 1987; Araya et al., 1989). Studies have shown that Interleukin-10 gene promoter polymorphisms can affect production of these cytokines (Cheers, 1984; Jiang and Baldwin, 1993; Jones and Winter, 1992; Baldwin and Parent, 1992). In a study conducted in 2008 in Iran, the effect of polymorphisms on susceptibility to brucellosis disease was examined. In the former study, 190 patients with brucellosis and 81 healthy ranchers who had infected animals and consumed infected dairy products were studied. All patients were genotyped in terms of two allelic polymorphisms in interleukin-10 gene promoter region at positions of 1082 (G/A), 819 (T/C) and 592 (A/C) using PCR-RFLP. The research results showed that distribution of CC genotypes and C alleles in positions of 592 and 819 of IL-10 gene were significantly higher in patients compared to healthy subjects (P-value was respectively equal to 0.034 and 0.008). Thus, ATA single and double haplotypes were significantly higher in control group compared to patients (P-value were respectively as 0.0278 and 0.013). Therefore, higher frequency of C alleles at positions of -819 and -592 of IL-10 and lower frequency of ATA/ATA haplotype in patients are considered as predisposing factors for brucellosis disease (Rasouli et al., 2009).

Fernandes et al. also conducted a study and showed that neutralizing IL-10 by monoclonal antibodies produces gamma interferon. In addition, lysing power of spleen cells increases against Brucella Abortus (Fernandes et al., 1995). Down-regulation effects of IL-10 in immune system of the subjects infected with intracellular bacterial and parasitic infections were also observed (Bermudez et al., 1993; Wagner et al., 1994; Sher et al., 1991; Silva et al., 1992).

Molecular methods for diagnosis of brucellosis were also conducted. Khamesipour et al. conducted brucellosis molecular diagnosis on 135 blood samples from slaughtered sheep using polymerase chain reaction. Then, their DNA was extracted and isolated using PCR method for diagnosis of brucellosis. In total, 135 samples were studied in which 42 cases (31.11%) were infected with brucellosis. These were diagnosed positive in PCR tests (Khamesipour et al., 2013). In another study conducted by Sharifi-Zadeh et al. brucellosis and leptospirosis were molecularly diagnosed in abortion cases using multiplex PCR. In this study, multiplex PCR method was adjusted for simultaneous searching of these two bacteria. Then, these factors were directly determined from contents of aborted bovine fetuses. It was both simplicity and possible to search simultaneously for these two bacteria. Then, multiplex PCR method can be used as a convenient alternative culture method compared to conventional methods (Sharifi-Zadeh et al., 2010).

Raeisi et al. conducted a study in 2010. In this study, an indirect ELISA kit was designed for serological diagnosis of brucellosis and achieving higher sensitivity compared to other conventional methods. In this study, smooth lipopolysaccharide (S-LPS) of Brucella melitensis prepared commercially with high purity was used as antigen to coat microplates. Indirect ELISA method showed positive results in 194 serum samples among which 194 samples showed positive Wright test using the designed ELISA kit while 72 samples showed negative results from 75 negative serum samples. These respectively indicated 100% sensitivity and 96% specificity for the
designed kit. Accordingly, the threshold or cut off was determined as 0.19. This test showed high accuracy and speed in conducting the test compared to other serologic tests and foreign kits (Reeisi et al., 2010).

Pakzad et al. conducted a study conducted in 2011 on 700 blood samples collected from febrile patients with suspected brucellosis who visited Ilam hospitals and laboratories for serological tests. These samples were screened using Rose Bengal Test. Then, 50 positive Rose Bengal samples were examined by Wright, Combs Wright tests and PCR using two genes of 16srRNA and L7/L12 while 50 negative Rose Bengal samples were tested using PCR with two aforementioned genes. The results indicated that 125 samples were positive while 575 were negative among 700 samples tested by Rose Bengal. In addition, 50 Rose Bengal positive samples were positive in PCR using both genes while 50 Rose Bengal negative samples were negative in PCR using both genes. Moreover, 47 samples in Wright test and 49 samples in Coombs Wright test had high titers of 1:60. These researchers found out that PCR method has higher sensitivity and specificity compared to serological methods in human brucellosis diagnosis. PCR has similar sensitivity as 16srRNA gene using L7/L12 genes. It can be used for human brucellosis diagnosis (Pakzad et al., 2011).

CONCLUSION

Brucellosis is one of the most common diseases among human and animal, which is called thousand faces disease due to long lasting side effects. Certainly, identification and control of disease transmission methods can promote public health. No effective and safe vaccine is available for humans. Clinical signs alone are not sufficient for brucellosis diagnosis. Then, a sensitive, specific, rapid and inexpensive method is required. Early and appropriate diagnosis of this disease is effective in improving public health as well as disease control and eradication. Several serological tests for probable diagnosis of Brucella infection were used in evaluation of antibodies against Brucella. The oldest tests are tube standard agglutination test or Wright Test, Coombs Wright methods, anti-human globulin test, complement fixation test (CFT), agglutination 2 – mercaptoethanol (2-ME), Rose Bengal Test and rapid agglutination on lam. These are serological diagnosis methods for brucellosis. These are not appropriate for definitive diagnosis of brucellosis. Using new methods such as Elisa has higher sensitivity and specificity than standard SAT test and complement fixation, which can show both G and M immunoglobulins. It is also suitable for examining certain class of immunoglobulin. On the other hand, this method shows all antibodies generated in reaction with surface antigens of Brucella. It can also prevent the complexity created by glucan or incomplete antibodies. Therefore, acute brucellosis can be easily diagnosed from chronic brucellosis using this method. When interpreting agglutination test is met with confusion, the result can be confirmed using ELISA test. Although SAT method has relatively high sensitivity, it is time-consuming. The results should be read with focus and precision. However, ELISA is one of the methods for in vitro measurement of immune response in the solid phase. Therefore, many drawbacks of safety evaluation methods in liquid phase such as time-consuming manner, initial preparation and high non-specific connections are eliminated in this new method. It should be noted that IgG, IgM (IgG1, IgG2), IgA and partial amount of IgE are produced in Brucellosis humoral immunity response. IgG is particularly involved in serological tests. IgM appears on the fifth to seventh day of brucellosis infection and reaches the final amount during 13 to 21 days after bacteria penetrated the body. Low amount of IgA is also generated in the interval between emergences of above two immunoglobulins. IgG titer is higher and more durable during the disease. This is significant in serological survey of brucellosis when the serum is tested. If infected serum in the first week was tested, no immunoglobulin may be observed. Thus, the test result will be negative. IgM level increases in second week. IgG is generated between the second and third weeks. IgG reaches the maximum level after three weeks. This level is still high during infection. Research and studies have shown that ELISA is a complete method for in vitro detection of chronic samples, especially when other tests results are negative. In addition to this method, all unique and specific immunoglobulin in tested serum appear with high speed and accuracy. In recent years, indirect ELISA has considerably improved. In most experiments, a purified variable amount of S-LPS is used as antigen. Another diagnostic method is PCR, which has higher sensitivity and specificity in comparison with serologic methods for diagnosis of human brucellosis. PCR shows similar sensitivity as 16srRNA using L7/L12 gene. It can be used in diagnosis of human brucellosis. Another diagnostic method is identification of different forms of IL-10 gene, which is a cytokine. It inactivates macrophages and infects the susceptible subject with brucellosis. Therefore, identification of different forms of IL-10 gene, which affect production of these cytokines, is considered as effective method for diagnosis of the disease. It is concluded that due to limitations of serological and culturing methods, which are time consuming, risky and expensive, as well as importance of early detection of bacteria in epidemic cases, it is recommended to use this new and effective method because many of these methods can overcome limitations of traditional methods.

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