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Case report

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## Multipronged diagnostic approaches for monitoring the treatment of *Brucella abortus* infected patient: a case report

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## ABSTRACT

Brucellosis caused by *Brucella* species is readily transmissible to humans, causing acute febrile illness and undulant fever which may progress to a more chronic form and can also produce serious complications affecting the musculoskeletal, cardiovascular, and central nervous systems. A veterinary livestock inspector presented to the institute with symptoms of intermittent fever, pain involving muscles and joints, loss of weight, anxiety and weakness for about three months has been investigated. The isolation, serological tests and PCR were performed for diagnosis of brucellosis. Based on history of constant professional association with animals, characteristic symptoms, hematological and biochemical, multiple serological and PCR assay results, the patient was diagnosed as brucellosis. Detection of *Brucella abortus* directly in the clinical samples by gel based PCRs were highly useful for diagnosis and monitoring of treatment. This diagnostic protocol will facilitate in a simple way to map major *Brucella* species infecting humans in a geographical region.

### 1. Introduction

Brucellosis is a second most important zoonosis of global importance after rabies, caused by intracellular Gram-negative coccobacilli of the genus *Brucella*. Brucellosis has been an occupational risk for farmers, veterinary surgeons and slaughter house workers[1]. Non-occupational sources of infection include consumption of fresh unpasteurized cheese and milk[2]. *Brucella* may also be transmitted from inhalation of aerosols. Four species of *Brucella* are the primary causes of infection in humans. *Brucella melitensis* (*B. melitensis*) is highly infectious and is transmitted from sheep and goats, *Brucella abortus* (*B. abortus*) from cattle, *Brucella suis* from pigs and infrequently *Brucella canis* from dogs. Other species of *Brucella* have been rarely or not reported to infect

humans.

The disease manifests with diverse symptoms such as fever, sweating, malaise, anorexia and headache, joint and back pain. Accurate diagnosis of brucellosis demands systematic study of epidemiology, clinical symptoms, biochemical and hematological profiles because it overlaps with many infections due to *Salmonella*, *Yersinia* and *Vibrio*[3]. Though brucellosis is diagnosed in the laboratory by various techniques like cultural, serological and molecular methods, the timely and accurate diagnosis of human brucellosis continues to be a challenge to clinicians because of its non-specific clinical features, slow growth rate in blood cultures and the complexity associated with serodiagnosis[4,5].

Confirmatory diagnosis requires isolation of brucellae from blood, bone marrow or other tissues of the patient. However, isolation is time-consuming and hazardous to laboratory workers. Hence, combination of two or more serological tests are preferred to avoid false positive and negative results. Recent improvements have made it possible to amplify DNA targets through different PCR methods instantly abbreviating the time required for multiple tests. The present report emphasizes on multiple diagnostic approaches for monitoring the treatment of *B. abortus* infection by isolation, serological tests and PCR in a clinical case.

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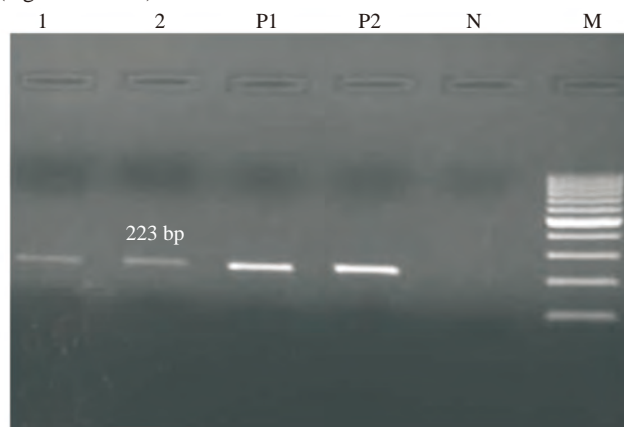
## 2. Case report

A 46 years old veterinary livestock inspector was reported to the institute with symptoms of intermittent fever, pain in muscles and joints, loss of weight, anxiety and weakness for about three months. He has been actively involved in artificial insemination of cattle as part of the cross-breeding program undertaken by the Department of Animal Husbandry, Government of Karnataka, India. He was treated at many hospitals with various analgesics, antipyretics, antibiotics and other supportive medications for pyrexia of unknown origin before reporting himself for brucellosis investigation. Hematological investigation reports showed that the patient had nearly normal blood cell count with relative lymphocytosis, mild anemia and elevated C reactive protein level. He was negative for typhoid, malaria, hepatitis B virus, tuberculosis, dengue, HIV and leptospirosis as per the tests performed in health care laboratories. Based on the characteristic symptoms, history of close contact with cattle and non-responsive to the various treatment regimen over a period of three months, the patient was suspected for brucellosis and referred to the institute for diagnosis. Informed consent was taken from the patient for the collection of blood before and after 4, 6 and 12 weeks of post treatment. Multiple diagnostic tests such as isolation, serological and molecular tests were performed to guide the patient for proper treatment and recovery.

First, isolation of *Brucella* was performed using fresh blood of patient directly into Castaneda's biphasic media prepared from *Brucella* selective agar and broth (BD BBL™) with supplements (Oxoid) as per the standard procedure[3]. Simultaneously, the serum samples were subjected to series of serological tests like rapid screening rose bengal plate test (RBPT). RBPT positive serum samples were further evaluated by serum agglutination test (SAT) and 2-mercaptoethanol agglutination test (2-ME SAT). The immunoglobulin G (IgG) and immunoglobulin M (IgM) based indirect ELISAs were performed using smooth lipopolysaccharide antigen from *B. abortus* S99 and anti-human IgG and IgM conjugated with horse radish peroxidase[6]. In-house developed lateral flow assay (LFA) using smooth lipopolysaccharide antigen and colloidal gold-protein G conjugate for binding vast range of IgGs irrespective of the species of livestock and human developed in collaboration with ubio Biotechnology Systems Pvt. Ltd. were used. A genus specific *Brucella* cell surface protein (*bcsp* 31) PCR was performed using the primers[7], and another set of IS711 genus and species specific PCRs suitable to use with both conventional and real time PCRs were carried out as per the standard procedure[8].

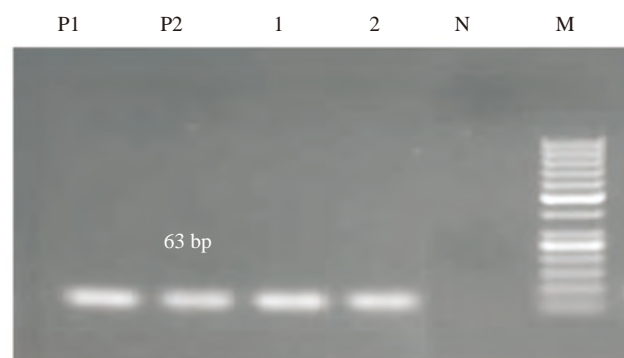
Isolation could not be made from the patient blood samples tested before the initiation of treatment and after three different intervals of treatment. Whereas, serum sample collected before initiating the treatment was positive by all serological tests such as RBPT, LFA, IgM and IgG ELISA with 1:640 and 1:160, SAT and 2-ME SAT test titres, respectively. Post treated samples were positive by RBPT, LFA and IgG ELISA and reduction in the antibody titres were observed in SAT (1:80) and 2-ME SAT (1:20) titres with reduced optical density values in IgM ELISA (Table 1). The blood and serum DNA from samples taken prior to the treatment amplified 63 bp and 223 bp products in IS711 and *bcsp* 31 genus specific PCRs, respectively, confirming presence of *Brucella* DNA. Similarly, amplification of 81 bp product in species specific PCR (BruA2\_0168 primer) indicated *B. abortus* (Figures 1, 2 and 3). The post treated blood and serum DNA samples were negative for *Brucella* genus and species specific PCRs

(Figures 4 and 5).



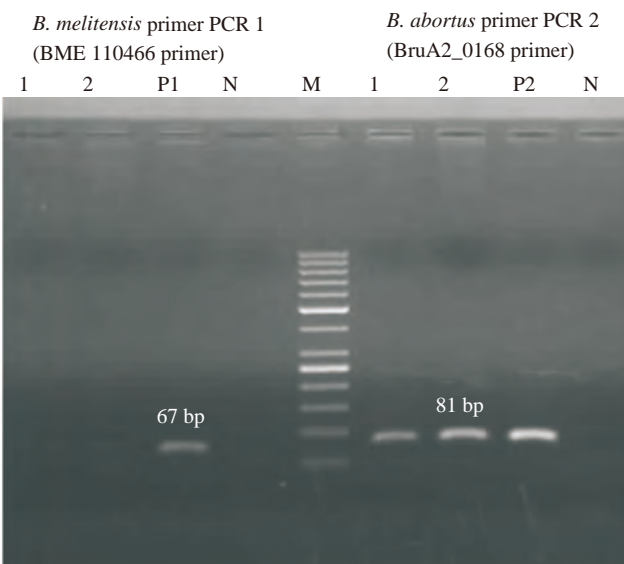
**Figure 1.** *Brucella* genus specific *bcsp* 31 PCR for pretreatment clinical samples.

Lane 1: Serum DNA; Lane 2: Blood DNA; Lane P1 and P2: Positive controls (*B. melitensis* 16M and *B. abortus* S99); Lane N: No template control; Lane M: 100 bp ladder.



**Figure 2.** *Brucella* genus specific IS711 PCR for pretreatment clinical samples.

Lane 1: Serum DNA; Lane 2: Blood DNA; Lane P1 and P2: Positive controls (*B. melitensis* 16M and *B. abortus* S99); Lane N: No template control; Lane M: 50 bp ladder.



**Figure 3.** *Brucella* species specific PCR for pretreatment clinical samples.

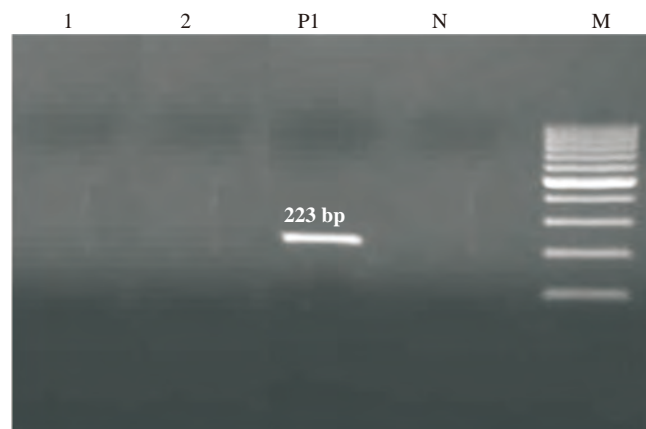
Lane 1: Serum DNA; Lane 2: Blood DNA; Lane P1 and P2: Positive controls (*B. melitensis* 16M and *B. abortus* S99); Lane N: No template control; Lane M: 50 bp ladder.

**Table 1**

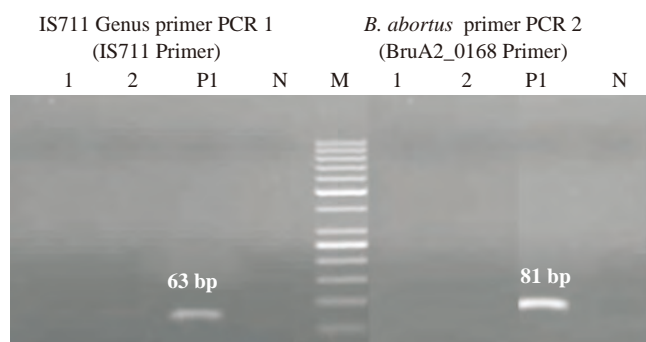
Results of multiple diagnostic tests of clinical samples collected at different intervals.

Samples at different intervals	Serological tests						Molecular tests			
	RBPT <sup>#</sup>	LFA <sup>#</sup>	SAT (titre)	2-ME SAT (titre)	IgM ELISA (OD) <sup>*</sup>	IgG ELISA (OD) <sup>*</sup>	<i>bcs</i> 31 genus PCR	IS711 genus PCR	<i>B. melitensis</i> specific PCR (BME110466)	<i>B. abortus</i> specific PCR (BruA2_0168)
Pre treatment	+++	++	1:640	1:160	1.2	1.4	+	+	-	+
4 weeks after treatment	++	+	1:320	1:80	0.9	1.2	+	+	ND	+
6 weeks after treatment	+	-	1:80	1:20	0.5	1.1	-	-	ND	-
12 weeks after treatment	+	-	1:80	1:20	0.3	0.9	-	-	ND	-

OD: Optical density; <sup>\*</sup>: Optical density above 0.7 was considered positive for both IgG and IgM ELISA; +: Positive result; <sup>#</sup>: Represents grading of positive (+) results; -: Negative test result; ND: Not done.

**Figure 4.** *Brucella* genus specific *bcs* 31 PCR for post treatment clinical samples.

Lane 1: Serum DNA; Lane 2: Blood DNA; Lane P: Positive controls (*B. abortus* S99); Lane N: No template control; Lane M: 100 bp ladder.

**Figure 5.** *Brucella* genus specific IS711 and species specific PCR for post treatment clinical samples.

Lane 1: Serum DNA; Lane 2: Blood DNA; Lane P1: Positive controls (*B. abortus* S99); Lane N: No template control; Lane M: 50 bp ladder.

Based on the panel of serological and molecular test results, the patient was confirmed to be suffering with brucellosis caused by *B. abortus* sp. He received specific treatment recommended for brucellosis (doxycycline capsule 100 mg twice a day; rifampicin capsule 600 mg per day for 8 weeks). The treatment was discontinued after six weeks and patient did not report any symptoms of joint and muscular pain, fever *etc.*, and had decreased anxiety and expressed willingness to resume his work.

### 3. Discussion

It is well understood fact that the greater risk for brucellosis is observed among veterinary doctors, veterinary technicians, inseminators, zoo workers, farmers, cattlemen and employees of

meat processing enterprises. Because of lack of human vaccines and effective control measures in livestock, it is necessary for the veterinary doctors and other health care workers to use protective measures like gloves and masks while handling animals to reduce occupation-related brucellosis[1]. In the present investigation, the patient was involved in bovine artificial insemination program and had constant exposure to brucellosis infected cattle. The patient confessed that he has ignored basic biosafety measures at times and probably it was presumed to be the main cause of the infection. He has reported symptoms like osteoarticular and muscular pains, fever and other associated symptoms except pain in the testicles. Persistence of symptoms even after treatment with various antibiotics over a period of three months was reported by the patient. Overlapping symptoms, consulting different physicians in short period of time, lack of awareness about the disease, compounded with limited access for laboratory testing for brucellosis resulted in delayed diagnosis.

Isolation could not be obtained from blood samples collected from patient even before and after treatment intervals. Also the patient had undergone treatment with various antibiotics before reporting for investigation. The failure to isolate *Brucella* could be due to low recovery and poor growth rate of *Brucella* in antibiotic treated chronic cases[9]. In the absence of isolation, serological tests are commonly used and the tests depend largely on the detection of IgG and IgM in the serum. The patient serum sample showed a titre of  $\geq 160$  which is considered positive SAT titre and more precisely 1:320 as more specific cut-off in brucellosis endemic India[10].

Agglutinating antibodies mainly from immunoglobulins of the IgM class occur in acute and subacute forms of brucellosis in humans. Total disappearance of agglutination reaction indicates IgM class and to the contrary, the lack of the effect of reducing the titre after 2-ME reduction is evidence of IgG class[11]. Hence assessment of the stage of infection by SAT with and without 2-ME SAT is essentially required in panel of diagnostic tests used for brucellosis as well as to monitor treatment follow up cases. The reduction in antibody titres in post treated samples was a clear indication of reduction of active infection.

Serological techniques used in the diagnosis of brucellosis have problem of false positive and false negative results in many instances[5]. However, serology remains the mainstay of laboratory diagnosis and large number of techniques in use are the evidence of the problem[12]. However recently, PCR assays have been extensively used in conjunction with serological tests for diagnosis of brucellosis[13]. In humans, the disease is caused mainly by *B. melitensis* as the most pathogenic species, followed by *Brucella*

*suis*, whereas *B. abortus* is considered as the mildest type of pathogen for brucellosis[14]. As per the history of patient who had exposure to cattle, the possibility of *B. abortus* and *B. melitensis* species infection was presumed and accordingly, PCR reactions were performed using *B. abortus* and *B. melitensis* specific primers. The genus specific *bcs* 31 primer has been widely used for confirmation of cultures and clinical samples for brucellosis[14-20]. However, the suitability of species detection by PCR in clinical samples has been explored in the present investigation. The results of genus and species detection by conventional PCRs for reference, field and non-*Brucella* strains were well correlated with results of probe based real time PCR[8]. Detection of *B. abortus* in clinical samples by simple gel based uniplex PCRs was found interesting and useful for routine, faster and accurate diagnosis. The identification of *Brucella* upto species level in infected human clinical samples was major constraint in diagnosis of brucellosis. This detection and mapping of *Brucella* species in humans of a geographical region is of great value in epidemiology of human brucellosis. Additionally, LFA tests, yet another field based test similar to RBPT is highly suitable for peripheral diagnostic laboratories and if made available to health care centers will facilitate quicker diagnosis of human brucellosis.

Testing for anti *Brucella*, antibody and genome by genus and species specific PCRs were very useful for declaring active infection status of the patient. Combination of doxycycline and rifampicin for six weeks facilitated faster recovery of the patient. Reduced antibody IgM titres and negative PCR results in post treated samples confirmed successful therapy. In the absence of isolation, various sero diagnosis approaches coupled with detection of *Brucella* genus and species specific gel based PCR assays appear to be promising for accurate diagnosis and follow up of treatment efficacy for brucellosis.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgments

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