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Research Article

Detection of *Brucella* Species from Seropositive Animal Blood Clots using real time PCR assay

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- Blood clot; *Brucella*; Cattle; DNA extraction; PCR assay; Small ruminant

Abstract

Brucellosis is a zoonotic disease transmitted from animal to human. It is economically important diseasecausing abortion, sterility and still birth in adult animals. The diseases can be diagnosed using serological, bacteriological and Molecular methods.The study was conducted to detect *Brucella* species from blood clot of C-ELISA positive samples using Real Time PCR. Blood sample collected and blood clot separated from collected blood samples. Serum sampleswere screened using Rose Bengal Plate Test finally confirmed by C-ELISA. Blood clot of C-ELISA positive blood clot of cattle and small ruminant subjected to PCR assay to detect *Brucella* species. Bacterial DNA was extracted according to Qiagen DNA extraction kit standard procedure and amplified. Species specific primers for *B. abortus* and *B. meltensis* were used to detect *Brucella* species from extracted DNA. *Brucella abortus* was detected from blood clot of cattle but from blood clot of small ruminant neither *B. meltensisnor B. abortus* detected. Molecular methods accompanied with screening and confirmatory serological testsare more suitable method to detect *Brucella* species than bacteriological method in developing countries; where Biosafety equipment or materials are not adequately available for safety of lab personnel's. Therefore, laboratories should be capacitated for molecular method of diseases diagnosis and investigation.

INTRODUCTION

Brucellosis remains widespread in livestock populations, and represents a great economic and public health problem in African countries and worldwide [1]. Brucellosis is a contagious disease of human and animals caused by bacteria of the genus *Brucella*. Brucellosis has a worldwide distribution and affects cattle, sheep, camel, goats, pigs dogs and, occasionally, horses. *Brucella* infections have also been recognized worldwide in a great diversity of wildlife species and, more recently, in aquatic mammals [2-4].

In human, the disease, which is often stated as 'undulant fever' and 'Malta fever' is a serious public health problem with involvement of different tissue, organ or system complications [2,5,6]. Brucellosis remains one of the most common zoonotic diseases worldwide, with more than 500,000 new cases in human annually where as in animals it is stated as infectious abortion and Bang disease [7,8].

In Ethiopia, farmers, agro-pastoralist and pastoralist have close contact with their animals.Their interaction is highly bonded together in daily life (while milking, during parturition and consumption of animal product) of the community leading them to get infection from positive animals [4,9,10]. The farmers in high land area of the country mainly use animals for draught power and income generation to lead their life. In pastoral area, pastoralist's daily life fully dependent on livestock and livestock product for livelihood whereas agro-pastoralists partially support their livelihood by cultivating of land for crop production in addition to their livestock production [4,9].

Different testes can be used for Brucellosis diagnosis in animal and human.Diagnostic tests fall into two categories: those that demonstrate the presence of the organisms and those that detect an immune response to its antigens [2, 11]. The isolation of *Brucella* is definitive proof that the animal is infected, but not all infected animals give a positive culture; the methods and facilities that must be employed are not always readily available [2,12]. The detection of antibody or a hypersensitivity reaction provides only a provisional diagnosis, but in practice it is the most feasible and economic means of diagnosis [2,13,14].

Real-time PCR assays have been described in order to test *Brucella* from urine, blood paraffin-embedded tissues and milk [15-18]. The primers used in these real-time PCRs derived from the insertion element, IS711 whereas the reverse primer and FRET probes are selected from unique species or biovar-specific chromosomal loci [16].

The isolation and identification or detection of *Brucella* offers a definitive diagnosis of brucellosis. However, attention should be given for minimum standards requirements of laboratory safety for handling *Brucella* [2,19]. Since Ethiopia is one of developing countries in African content, both human and veterinary laboratories are not equipped properly to test the diseases in

safe way laboratory procedures. Therefore, the objective of study was to detect *Brucella* species from seropositive cattle and small ruminant (sheep and Goat) in study area using molecular assay.

MATERIAL AND METHODS

Sample collection

Blood sample were collected from cattle and small ruminant in selected district of Borena zone Oromia regional state, Ethiopia. Blood clot was separated from serum properly in Yabello regional veterinary laboratory and stored in -20 refrigerators. Corresponding positive serum blood clots subjected to molecular assay.

Laboratory tests

Serological tests: After sera samples were screened by RBPT and confirmed by c-ELISA the blood clots for which its corresponding serum samples were c-ELISA positive subjected to real time PCR.

Molecular Methods (Real Time PCR)

Extraction of DNA from Blood clot for PCR assay: Bacterial genomic DNA Extraction was made from eighteen (18) blood clot; six (6) shoat blood clot and twelve (12) cattle blood clot of seropositive samples using the Qiagen DNA extraction kit. Qiagen DNA extraction kit uses Internal Positive control (IPC) for screening of *Brucella* at genus level by Insertion Sequence 711(IS711) real time PCR. It combines RNase P PCR, for master mix (20µM Primer F, 20µMPrimer R, 20µM, Taq Man Universal PCR Master mix(2x), 10x Exo IPC Mix, 50x Exo IPC DNA and Water) for screening test of *Brucella* at genus level. The nucleotide sequence of forward and reverse primers used for screening were

5'-GCT-TGA-AGC-TTG-CGG-ACA-GT-3'and 3'-GGC-CTA-CCG-CTG-GGA-AT-5' respectively. For species specific real time PCR, species specific primers used for *B. abortus* nucleotide sequence were 5'-GCA-CAC-TCA-CCT-TCC-ACA-ACAA-3' and 3'-CCC-CGT-TCT-GCA-CCA-GACT-5'for forward and reverse, respectively. Similarly, species specific primers for *B. melitensis* nucleotide sequences of forward and reverse primers were5'-TCG-CAT-CGG-CAG-TTT-CAA-3' and 3'-CCA-GCT-TTT-GGC-CTT-TTCC-5' for forward and reverse primer respectively.

Amplification of DNA and Detection of Brucella species: An extracted DNA was amplified as manufacturer specific primers, probes and internal positive control (IPC). Primers, probe and internal positive control (IPC) was additionally added for screening test. The master mix components were made for three purposes in micro centrifuge tube separately for IS711, *B. abortus* and *B. meltensis.* The Insertion Sequence 711(IS711) was for screening test of *Brucella* genus in amplified DNA whereas the other two was made for *Brucella* species detection. Eighteen (18) blood clot samples twelve (cattle blood clot) and six (small ruminant blood clot) which its corresponding serum was positive by c-ELISA were used for species detection. Out of eighteen (18) extracted DNA, twelve (12) were from cattle whereas six (6) were from shoat. All extracted bacterial DNA from cattle and shoat blood clot allowed to amplify and selectively bind with *B. abortus* primers and *B. meltensis* primers to identify presence of *B. abortus* and *B. meltensis* separately. The thermal cycler was run 95°c for 10 minute to denaturation double stranded DNA, then amplification/extension occurred at 95°c for 15 second and 60°c for 1 minute. This process adjusted to run for 45 cycles. Finally,

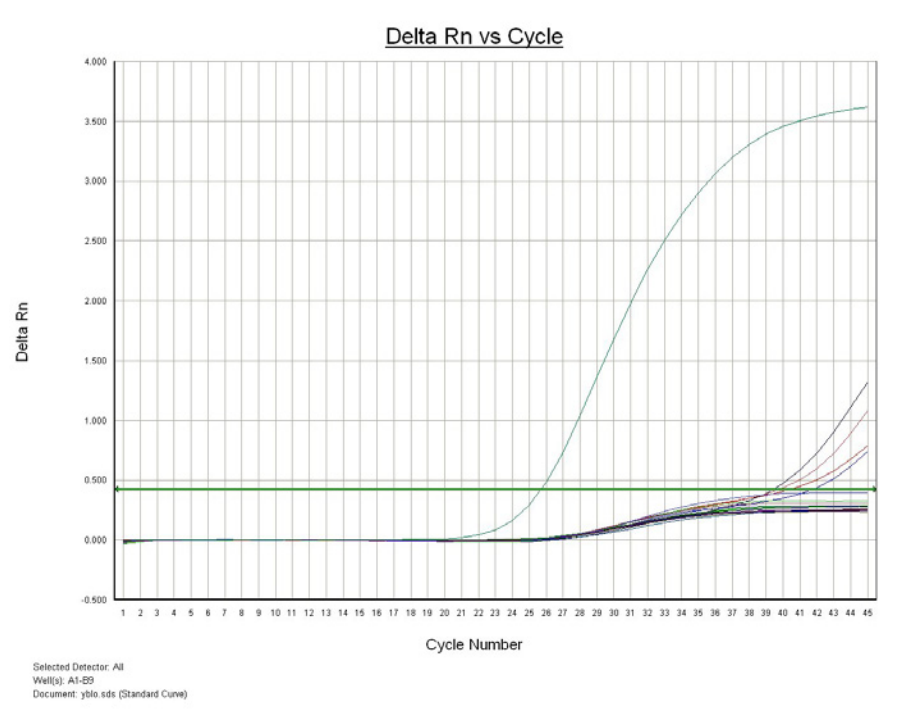

Figure 1 Graphical indication of real time PCR of screening test IS711 for presence of Brucella genus in amplified DNA.

Brucella species was detected using species specific primers of *B. abortus* and *B. meltensis* when the cycle threshold (CT) value of the samples were < 45, it considered and evaluated as positive. If greater than 45 it is considered as negative.

RESULT

All bacterial DNA extracted from blood clot of cattle allowed to amplify and specifically bind with *B. abortus* and *B. meltensis* primers. Out of eighteen (18) samples of extracted bacterial DNA, only two 2 (16.7%) samples from blood clot of cattle were shown *B. abortus* presence. However, from blood clot of small ruminants neither *B. abortus* nor *B. meltensis* was detected (Figure 1-3).

DISCUSSION AND CONCLUSION

In current study, *B. abortus* was detected from extracted DNA of C-ELISA confirmed blood clot of seropositive cattle using species-specific primers by real time PCR. Primers used were *B. abortus* species specific and *B. meltensis species* specific primers. However, *B. melitesis* was not detected using this method from both blood clot of cattle and small ruminant. Fail to detect *B. melitensis* from small ruminant blood clot could be due to absence of bacterial DNA in sample or absence of bacteria in blood clot that taken and subjected to DNA extraction and amplification. This is in agreement with corbel that absence of the bacteria not indicator for negativity of the animal because not all infected animals give a positive culture [2]. In another way, PCR can result in false negativity according [18]. In addition, the negative result especially, for *B. meltensis* in small ruminant blood clot could be due to less number of seropositive samples from small ruminant. The result obtained (*B. abortus*) from cattle blood clot is in agreement with previous study document that *Brucella abortus* is species of *Brucella* that responsible for causing abortion in cattle [1,2,20-22].

Fail to detect *Brucella* species in all seropositive animal does not mean animals are negative for Brucellosis. Detected species is also economically important pathogen implying the need for

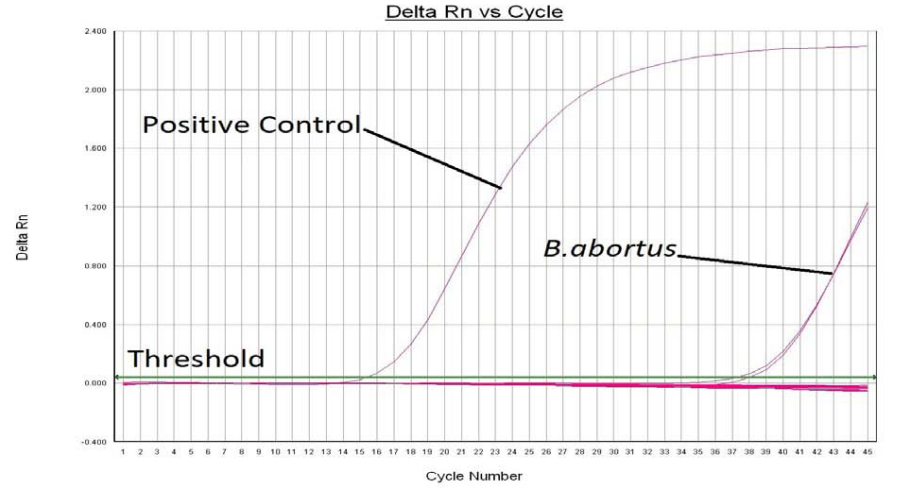

Figure 2 Positive result of *B. abortrus* from extracted bacterial DNA of cattle blood clot. CT Value =34.81 and 36.94.

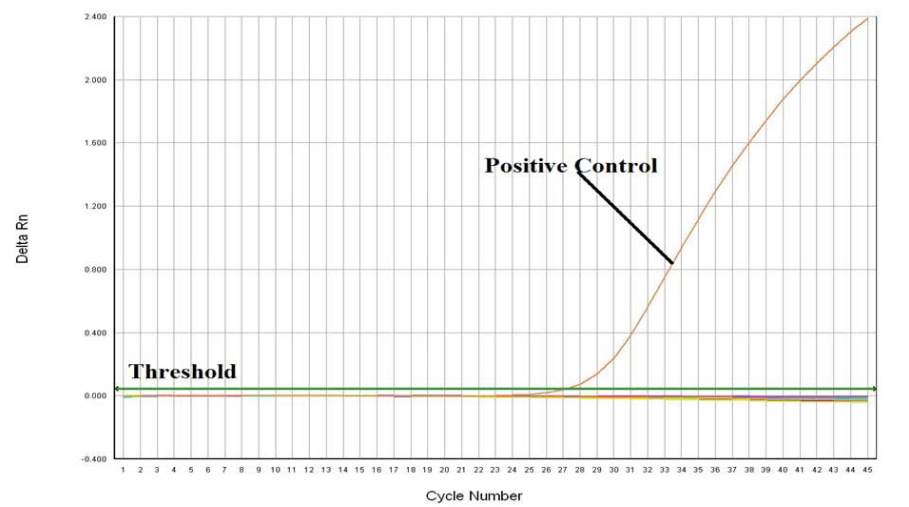

Figure 3 Negative result of small ruminant blood clot for B. melitensis showing negative result.

farther surveillance of Brucellosis and Isolation or detection pathogen in food animal in study area. Therefore, detection of *Brucella* species using molecular assay accompanied with screening and confirmatory serological tests in domestic animal is essential in study area where livestock and pastoralists have cross contact in their daily life to enable authorities or decision makers plan disease control and prevention strategies in future.

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