Journal of Animal and Veterinary Advances 8 (8): 1637-1640, 2009
ISSN: 1680-5593

Comparison of PCR Assay with Serum and Whole Blood Samples of Experimental Trials for Detection and Differentiation of *Brucella melitensis*

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Abstract: Brucellosis poses a significant animal and public health problem in many developing countries and requires fast and accurate diagnosis. A PCR assay amplifying part of the *Brucella melitensis* specific IS711 gene was developed and applied to mice clinical samples with experimental trial. Over an 8 week period of infection, whole blood and serum were examined from 78 experimental mice, with a total of 60 samples from *B. melitensis* infected mice and a group of 96 control samples from mice inoculated with *Brucella abortus* 544, *Yersinia enterocolitica* O:9 and *Brucella* broth. Regardless of date of infection, the sensitivity of whole blood and serum based PCR assay with samples from *B. melitensis* infected mice was found to be 100% (30/30) and 83.3% (25/30), respectively. Serum samples collected at 60 days post infection (p.i) of *B. melitensis* failed to show positive result. An amplicon of 252 bp was obtained in all PCR positive samples. All samples obtained from the control groups tested negative, conferring an assay specificity of 100%. These results show that though, use of serum-PCR may lead to assay simplification and shorten turnaround time, but the optimal clinical specimen for this test was not serum but whole blood, which leads to maximum assay sensitivity.

Key words: *Brucella*, PCR, serum, whole blood, mice, IS711

INTRODUCTION

Brucellosis is an infectious zoonotic disease that is associated with chronic debilitating infections in humans and reproductive failure in domestic animals (Leyla *et al*., 2003). *Brucella melitensis* with its three biotypes causes disease mainly in small ruminants and is the most pathogenic species for humans (Davidson *et al*., 1990). Currently, the diagnosis of brucellosis is mainly based on serological and microbiological tests. The former tests have the disadvantage of cross-reacting with other common antigentically related bacteria like *Yersinea enterocolitica* and do not detect early and latent infections. The later tests are cumbersome and of high risk to people carrying them out. As for other fastidious pathogens, molecular methodology offers an alternative way of diagnosing brucellosis. Nucleic acid amplification techniques, like PCR, characterized by high sensitivity and specificity and short turnaround time can overcome the limitations of conventional methodology. Only few studies in the literature (Amin *et al*., 1995; Leal-Klevezas *et al*., 1995) however, addressed direct detection of *Brucella* sp. in clinical specimens of animal origin.

The application of serum PCR for diagnosis of infectious disease has been reported by several investigators (Bougnoux *et al*., 1999; Morris *et al*., 1996). In brucellosis, however, as the microorganisms of the *Brucella* genus are facultative intracellular pathogens and the innoculum found in the host is normally small, most studies of PCR assays involving both human and animal brucellosis have been undertaken with whole blood samples (Leal-Klevezas *et al*., 1995). Although, Zerva *et al*. (2001) found sufficient DNA in human serum fraction to detect infection by *Brucella* but, its diagnostic value with different stages of infection has never been studied and compared with whole blood.

In this research, we studied the diagnostic utilities of serum over whole blood as suitable clinical samples using PCR with clinical samples obtained from experimental trials over an 8 week period of infection.

MATERIALS AND METHODS

Animals and experimental infection: Seventy eight female mice (8 weeks of age) obtained from University Putra Malaysia (UPM) laboratory animal section were acclimated and randomly distributed into five experimental groups. Group A: 18 mice infected with *B. melitensis* Malysian isolate (isolated from sheep); group B: 12 mice infected with *B. melitensis* 16 M (reference strain); group C: 12 mice infected with *B. abortus* 544 (reference strain);

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group D: 12 mice infected with *Y. enterocolitica* O:9 (reference strain). Mice were kept in conventional animal facilities and received water and food *ad libitum*.

All the test bacteria were first grown onto *Brucella* agar under appropriate condition and were used for subsequent experimental infection of mice. Briefly, from *Brucella* agar, single colony of each bacterium was transferred into 10 mL of *Brucella* broth and incubated at 37°C with agitation for 72 h (*Brucella*) and at 25°C for 18 h (*Yersinia*).

The concentration of bacteria in the broth was adjusted to 0.5 MacFarland turbidity standards and from which 1 mL, approximately containing 5×10^8 cfu was used to infect the first four groups of mice with the respective bacteria intraperitonially by the methods described previously (Zerva *et al*., 2001). In addition, 24 mice, each injected with 1 mL of *Brucella* broth were included in the experiment (group E) and used as a negative controls for each of the above four infected groups. Samples were collected and prepared for PCR tests over an 8-week period of infection.

Sampling: At 1, 7, 14, 30, 45 and 60 days following exposure, 3 mice (group A) and two mice (group B, C, D) and one mouse (group E) for each time-point were exsanguinated by intracrdial puncture for blood collection before euthanization All stages were conducted with consideration for their welfare.

Isolation of DNA: Whole blood samples from all experimental and control groups of mice were collected in EDTA and without anticoagulant. All samples were aliquoted and stored at -20°C until tested. A 0.5 mL portion of anticoagulated whole blood was mixed with 1 mL of erythrocyte lysis solution (320 mM saccharose, 5 mM $MgCl_2$, 1% Triton X-100, 10 mM Tris-HCl (pH 7.5)) and centrifuged at 15,000×g for 2 min. The cell pellet was then washed with 1 mL of water three times. DNA was isolated from serum (100 μL) and whole blood pellets with MasterPure™ Complete DNA and RNA Purification Kit (EPICENTRE, USA). The concentration and purity of the extracted DNA was determined spectrophotometrically (BioQuest, UK) according to the method described by Sambrook *et al*. (1989).

The PCR assay: *Brucella melitensis* sp. primers used in the amplification process were F (5'-catgcgctatgtctggttac-3') and R (5'-agtgtttcggctcagaataatc-3') targeting *B. melitensis* sp. IS711 (Redkar *et al*., 2001). The PCR reaction was performed in a total volume of 25 μL consisting the following: 1X standard *Taq* reaction buffer containing 1.5 mM $MgCl_2$, 2 mM of each dNTP, 0.4 μM of both forward and reverse primer, 1.0 U of *Taq* DNA polymerase, 200 μg mL^{-1} BSA and 1.0 μL of extracted DNA. Amplification was then performed with an initial denaturation step at 94°C for 15 min, followed by 35 cycles of 94°C for 1 min (denaturation), 58°C for 1min (annealing), 72°C for 2 min (extension) and a final extension at 72°C for 5 min. Positive controls were included in all tests and comprised of *B. melitensis* 16M DNA isolated from pure culture; negative controls were also included and contained all the elements of the mixture except template DNA. All samples were processed in duplicate. Finally 5 μL of the amplification reaction was taken and fractionated in 1.5% agarose gel containing 1 X TBE (100 mM Tris-HCl pH 8.0, 90 mM borinc acid, 1 mM Na_2EDTA), stained with ethidium bromide solution (0.5 μg mL^{-1}) and visualized under UV light.

RESULTS AND DISCUSSION

Of 156 total samples included in the study, 36 (23%) were collected from *B. melitensis* Malysian isolate infected mice, 24 (15.4%) were from each of *B. abortus* 544, *B. melitensis* 16M and *Y. enterocolitica* O:9 infected mice and 48 (30.8%) were from *Brucella* broth injected negative control groups. The total number of both serum and blood is equal, each with 78 (50%). Though none of the mice were found dead as a result of experimental infection, but here in this study overt clinical signs of brucellosis were noted at earlier stages of infection (extreme shivering, erection of hair coat, anorexia, dullness), which virtually have disappeared at later stages, almost one month p.i.

Results showed that when whole blood DNA templates were examined by PCR, an amplicon of 252 bp was obtained in 100% (30/30) of the tested samples using *B. melitensis*-specific IS711 primers at each time point of examination. However, only in 25 out of 30 serum specimens was the 252-bp band amplified making its sensitivity 83.3% (Fig. 1). All serum samples collected at 60 days p.i of *B. melitensis* failed to show detectable band. All whole-blood and serum samples obtained from the control groups tested negative with the IS711-PCR, conferring an assay specificity of 100%.

Considering the advantages of PCR, the fact that there are theoretically fewer *Taq* polymerase inhibitors in serum than in whole blood and the fact that the DNA extraction process is easier in serum than in whole blood, the diagnostic yields of serum and whole blood was compared with samples obtained from experimental trials. All samples except serum samples collected at day 60 p.i. showed detectable *B. melitensis* DNA in both whole blood and serum (combined sensitivity, 100%), but the assay sensitivity was higher with whole blood samples (100%) than with serum samples (83.3%).

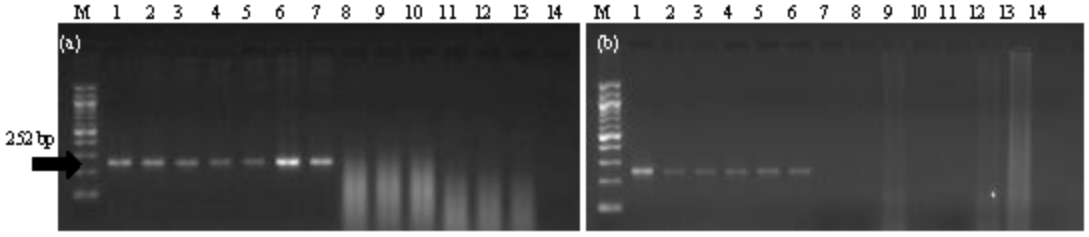

Fig. 1: Representative PCR amplification products of *B. melitensis* from whole-blood (i) and serum (ii) samples of experimentally infected mice collected at different dates of p.i. Lane M (100 bp DNA marker); Lane 1 (positive control); Lanes 2-7 (whole blood (i), serum (ii) samples collected at 1, 7, 14, 30, 45 and 60 days p.i from group A mice, respectively); Lanes 8-13 (whole-blood (i), serum (ii) samples collected at 1, 7, 14, 30, 45 and 60 days p.i from group E mice, respectively); Lane 14, NTC. An amplicon of 252 bp was obtained from positive samples

Several studies have documented the presence of circulating pathogen DNA in serum samples (Elfaki *et al*., 2005; Kawamura *et al*., 1999). These pathogenic nucleic acids in serum samples are most probably released into the circulation as breakdown products during bacteraemia. Zerva *et al*. (2001) also found sufficient DNA in the serum fraction than whole blood to detect acute human infection by *Brucella* using PCR method. In the present study, however, unlike the whole-blood PCR assay where all samples were positive at each time point of examination, the serum PCR assay detected positive samples of serum only until day 45 of p.i. This could be due to the reason that the amount of circulating DNA in serum at later/chronic stages of infection is presumably lower as a result of its being an intracellular pathogen but is still detected in whole blood. The presence of inhibitors in PCR-negative serum specimens was ruled out by re-examining samples diluted in water. In order to exclude the possibility of inefficient DNA extraction, aliquots of the original serum samples were thawed and DNA was re-extracted and used as a template for the PCR assay. The same results were obtained. The diagnostic sensitivities of the IS711-PCR assay for whole-blood and serum specimens thus corresponded to 100 and 83.3%, respectively. Though, PCR inhibitors were often detected in whole-blood specimens (Morata *et al*., 1998) but in this study, no inhibition was observed with any of whole blood samples tested showing that the DNA extraction technique has overcome problems associated with PCR inhibitors of clinical samples. This is especially relevant if we consider that, in the present study, the sample of mice with brucellosis was believed to be very representative of the true clinical situation, because it included both the acute and chronic phases of the diseases including latent stages.

The use of serum instead of whole blood samples may offer several advantages for nucleic acid amplification methods. Inhibition by anticoagulants, hemoglobin, host DNA, or any other substance present in whole blood but not in serum is circumvented. However, serious caution should be taken when serum-PCR instead of whole blood-PCR was used for intracellular organisms particularly for the genus *Brucella* whereby circulating DNA could be presumably low/absent at later/chronic stages of infection resulting false negatives. Leal-Klevezas *et al*. (1995) managed problems of PCR inhibitors with human whole blood samples using red blood cell lysis, repeated washings by centrifugation and measurement and adjustment of isolated DNA concentrations to the maximum dilutions.

CONCLUSION

The results show that though, use of serum-PCR may lead to assay simplification and shorten turnaround time, but the optimal clinical specimen for this test still was not serum but whole blood, which has lead to maximum assay sensitivity. It has often been reported that because of *Brucella* organism's being intracellular, the innoculum found in the host is normally small and brucellosis is characterized by a high degree of bacterial DNAemia mainly at earlier stages of infection (Queipo-Ortuno *et al*., 2004).

These results and facts together with our findings incline us to conclude that chronically infected animals presenting intermittent bacteremia, could more effectively be detected by whole blood-PCR than serum PCR. Although, the use of this whole blood-PCR assay in preference to serum-PCR as a supplemental diagnostic tool for detection and differentiation of *Brucella* organisms in clinical specimens could be recommend, the need for the assay to be validated with confirmed positive and negative clinical samples of its primary host animals and verify its ability to differentiate the vaccinal strains from field strains, is desirable to achieve the utmost benefits from the PCR assay.

ACKNOWLEDGEMENTS

The authors would like to thanks all staffs of Molecular Biology and Bacteriology laboratory of University Putra Malaysia for the excellent support through out the study.

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