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Comparison between conventional PCR and PCR - ELISA for detection of Brucella melitensis

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ABSTRACT

Molecular detection techniques are believed to be key tools for both prevention and treatment follow up of brucellosis within live stock and human beings. Consequently rapid, reliable, easy to perform and automated systems for Brucella detection are urgently needed to allow early diagnosis and adequate antibiotic therapy in time. Brucellosis is a worldwide re-emerging zoonosis causing high economic losses and severe human disease. In attempt to improve current molecular detection of Brucella melitensis, we compared a conventional PCR with PCR- ELISA, to detect brucella genome within standard strains and clinical isolates. Primer sets based on "omp-31" sequence of B. melitensis 16M were designed. The primer specificity was checked with appropriate online bioinformatics softwares. The primer specificity was also confirmed by testing the reaction with non-Brucella strains. A biotinylated probe complementary to an internal sequence of the PCR products was designed. The labeled non-specific fragments bound to streptavidin-coated wells, saturating the solid phase streptavidin by biotin-streptavidin interaction. Compared with conventional PCR, the PCR- ELISA proved to have more sensitivity for Brucella genome after appropriate optimization. Few human serum, whole blood and also different affected tissue samples from slaughtered livestock with brucellosis were used for protocol evaluation. Further samples should be tested before final conclusion about the results.

Key words: PCR – ELISA, Brucella melitensis, PCR

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1. Introduction

Brucellosis is a worldwide zoonosis that causes high economic losses and severe human disease. Brucellosis is a zoonosis with crucial importance to human and animal health. Infection in live stock is serious economically and play a key role in human illness (Corbel, 1997). Human brucellosis is a severe multi-organ disease that is mainly caused by infection with three different species of the genus; B. melitensis, B. abortus, and B. suis. Brucelosis is also a serious risk factor for regions where it is supposed to be eradicated due to increasing of tourism related activities in areas with high prevalence (Memish and Balkhy, 2004). Brucella spp. were among the first microorganisms weaponized in the 1950 decade, and still pose a threat as biological agents which could be used in bioterrorism attacks (Dahouk and Tomaso, 2004). Diagnosis is basically based upon cultural isolation followed by biochemical identification of bacteria or rising titers in serological tests. Common microbiological tests lack the necessary sensitivity/specificity, and are also time consuming. Human brucellosis is common in most of the endemic areas of the world but often not diagnosed.

Molecular methods are shown to have acceptable sensitivity and specificity for detection of the pathogen in clinical samples. PCR based assays holds much promise as key tools for the diagnosis of brucellosis in the near future (Bricker, 2002). Different PCR protocols had been previously developed and comparative studies has revealed different conclusion (Navarro and Escribano, 2002). Morata has reported that use of both conventional PCR and PCR – ELISA assay with whole blood samples provides better results compared with conventional microbiological techniques for both primary and relapsing brucellosis (Morata and Queipo-Ortuño, 1999). As a need for improvement of molecular based methods for assays of brucellosis with high efficiency, we developed an ELISA-PCR method using a new designed primer set based on outer surface protein 31-KD gene of B. abortus.

2. Methodology

2-1. Materials

2-1-1. Bacterial strains

B. melitensis 16M, B. melitensis vaccine strain RevI, B. melitensis biovar abortus strain 19, B. melitensis biovar abortus strain 99, B. melitensis biovar abortus strain 133, and B. melitensis biovar abortus strain 544 were used as standard strains. Culture of Brucella was performed using brucella agar (QUELAB) in 35oC for 3 days. S. aureus (ATCC 25923), E. faecalis (ATCC 25212), B. cereus (ATCC 9634), L. monocytogenes, L. pneumophila (clinical isolate), H. pylori (ATCC 26695), P. aeruginosa (ATCC 8821), N. meningitidis type B, K. pneumoniae (Clinical isolate), E. coli (ATCC 25922), S. typhimurium, were also used as non- Brucella strains.

2-2. Extraction of genomic DNA

The Brucella cells for DNA extraction were obtained from overnight culturing Brucella strains in Brucella broth (QUELAB) in a shaking incubator (Stuart, orbital incubator S150) at 35ºC. Non-Brucella cell density was also prepared by culturing them in nutrient broth. The specific Brucella broth were used for growing up the fastidious strains. AccPrep® Genomic DNA Extraction Kit (Bioneer) was used to extract genomic DNA. The human genomic DNA was also prepared from a healthy individual blood sample by means of same kit. All extracted DNA samples were submitted for spectrophotometric measurement of quantity and purity BioPhotometer, eppendorf®). The concentration and purity of the DNA were then determined spectrophotometrically by readings of A260 and A280.

2-3. Primer Designing

A pair of 22 and 24 nucleotide primers; N1: (5' GCA GTC AGA CGT TGC CTA TTG G 3'), N2: (5' AAT ATT TCT CCA GAA TCT TGT CCG 3') generating 428 bp products of the gene (nucleotide 789-1012), which encodes a 31 KDa protein of B. abortus antigen were designed. The bcs31 which conserved in all strains was selected for PCR amplification (Vizcaíno and Cloeckaert, 1996). GeneRunner 3.0 on the reference sequence with absolute inclusion of selected fragment was used for primer designing. Oligonucleotide primers were selected, using an appropriate computer program (Lowe, Sharefkin et al. 1990) from the published nucleotide sequence of a B. abortus gene that encodes a 31 KDa protein (Mayfield et al., 1988). A search of genbank and NCBI/BLAST sequence database did not identified any significant homologues with this Brucella sequence. The primers then synthesized, using Gene-Assembler Plus (Sina Gene Co).

2-4. DNA amplification

Conventional PCR was performed in a 20 μl mixture containing; primers (200nM for each; Sinna Gene Co.), dNTP mix (200μM for each dNTP), 1.5mM MgCl2, and Taq DNA polymerase 0.5 U/20μl total volume, using 50ng B. melitensis 16M DNA as template. DDW (Double Distil Water) with the same volume to DNA sample were used as negative control. Samples were cycled in a Thermocycler with an initial denaturation of 94ºC for 5min, followed by 35 cycles, denaturation at 90ºC for 1 min, predicted annealing at 58ºC for 1 min, extension at 72ºC for 1 min, and a final extension at 72ºC for 5min. PCR products (428bp) were assayed with agarose gel electrophoresis containing ethidium bromide 1μg/ml, using TBE buffer (pH 8.2) at 90v for 45 min.

2-5. Detection of PCR products by Digoxigenin-ELISA

Dig-ELISA was performed according the method previously described (Luk and Kongmuang, 1997; Morata and Queipo-Ortuno, 2003). The commercial streptavidin–coated plates (2.5 μg/ml) were used for Dig-ELISA reaction. Briefly, 5μl aliquots of the PCR products were mixed with 95 μl of dilution buffer (0.95% Tween 20 and 1% bovine serum albumin (PBS pH 7.2) containing, 5’ biotinylated oligonucleotide probe; 5’ GAA GAC GAT ATC AAG GCT G 3’ biotin-labeled probe (Roche Inc). Samples were added in duplicate to the wells of streptovidin-coated microtiter plates and incubated at 37°C for 2 h. A 100 μl aliquot of anti-digoxin Fab/peroxidase conjugates diluted 1:35000 in a peroxidase stabilizer buffer and was added to each well and incubated at 37ºC for 30 min. After washing step, with 200:l of PBS (pH 7.2) color was developed by addition of 100 μl of a ready-to-use liquid tetramethylbenzidine (Vircell SL) substrate. After 20 min incubation at room temperature in the dark, the color reaction was stopped with 50 μl of 0.5 M H_2SO_4 . The A450 nm of each specimen was measured in an ELISA reader. To define the detection limit of new developed PCR according to the amount of template DNA, extracted DNA of B. melitensis 16M has been diluted 10 times, and PCR was performed with diluted DNA samples through optimized procedure. This step was done as triplicate from template dilution to PCR. DDW was used as negative control.

A450 of each specimen was measured in an ELISA reader (MR-3100T; Dynex Technologies Inc., Chantilly, Va.) as a net value after subtracting the value for the blank. Each PCR-digoxigenin-ELISA was performed with positive (100 ng of DNA from B abortus B-19) and negative (distilled water) controls. All experiments were performed in duplicate, following contamination-free guidelines to prevent false-positive results. An assay was considered positive if the mean optical density value was more than three standard deviations above the mean value for the healthy controls. As the mean absorbance value and standard deviation for the 28 healthy subjects studied was 0.04-0.12, a sample was considered positive when the absorbance of the two measurements was greater than 0.3. In the event of discordant results between duplicate PCRs, i.e., one positive and one negative, the amplification procedure was repeated with a different extraction.

2-6. Statistical analysis

The SPSS 9.0 were used for statistical data analysis.

3. Result and discussion:

Gene encoding 31 KD protein that used for primer designing has been used as a popular target among research studies localized on brucella molecular detection (Morata and Queipo-Ortuño, 2003 ; Park and Lee, 2005; Queipo-Ortuño and Colmenero, 2005), except that in this work new primer designed to to improve the previous quality. In this study, a new PCR primer set was designed which amplifies a fragment of outer surface protein-31 gene and conserved in brucella strains. Previous experiences have shown that designing primers could be useful initially to try more than one pair, so we designed primarily three set. A primer pairs (N1, N2) were finally selected, after bioinformatics analysis.

PCR with N-1/N-2 primer pair appeared to be specific for Brucella and none of non-Brucella organisms or human genome showed any amplification. Restriction enzyme digestion by TaqI for all of Brucella amplified fragments yielded the same pattern and compatible to our predicted fragment lengths. Through optimization protocol it was found that MgCl2 at 2mM concentration and annealing temperature at 62oC for 45sec is the best for newprocedure. Denaturation duration and extension were found 45sec and 75sec respectively as an optimum according to the results. The detection limit of primer set and prob used in present study was 600fg genomic DNA (Fig. 1 and Fig. 2), equivalent to nearly 190 CFU according to previous studies (Casañas and Queipo-Ortuño, 2001).

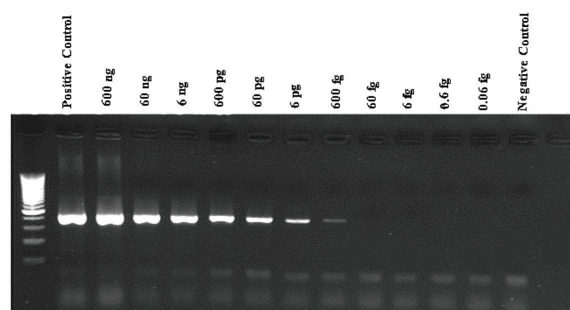

Figure1. *Detection limit of conventional PCR.*

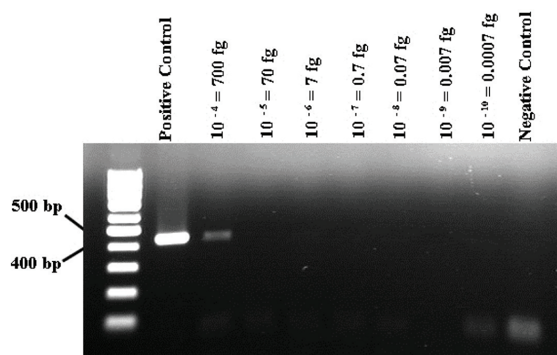

Figure 2. *Detection limit of primers in conventional PCR.*

Brucellosis as a zoonosis is hard to be detected in laboratory settings and with extensive economical looses annually, is a disease subjected to many of studies conducted to resolve the problems towards its detection. Assessment of fragment through TaqI digestion shows the specificity of our primer pair for the outer surface protein-31 gene fragment. PCR with the panel of standard Brucella strains and with the non-Brucella strains plus human genome in other experiment showed this procedure is highly specific for Brucella and non-Brucella DNA did not show any amplification. Also the detection limit is better than previous method and made more sensitivity. by considering that this PCR should be optimized for clinical experiments, but it shows valuable features by its potential for use in detection of the pathogen.

The available Brucella standard strains and clinical isolates were also tested. The absorbance of negative controls was below 0.5. but for positive control was above 0.5. The detection limit was determined by PCR amplification of a 10 fold serial dilution of a pure culture (Fig. 3).

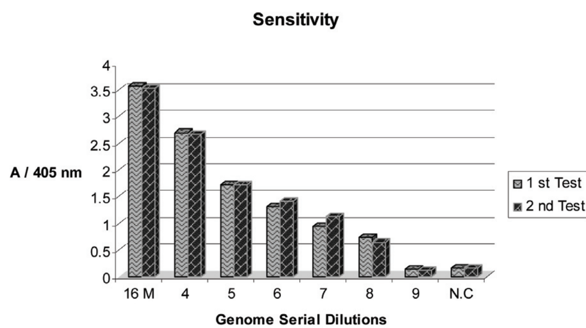

Figure 3. *Detection limit of PCR-ELISA.*

The samples of 12 clinical isolates were also applied to present PCR-ELISA system along with positive and negative controls, all were detected (Fig. 4)

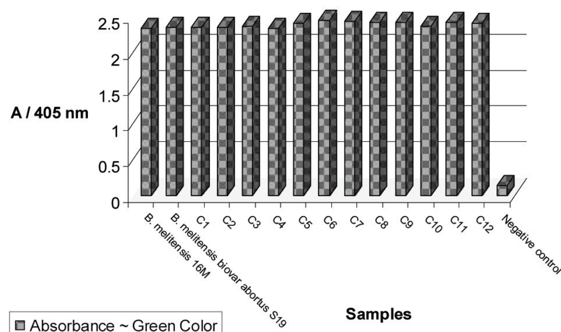

Figure 4. *Clinical isolates of Brucella.*

In the present study, the detection limit and primer specificity for were compared using conventional and PCR- ELISA techniques. The same target has been also used recently in order to molecular detection of Brucella genome (Newby and Hadfield, 2003; Probert and Schrader, 2004). Morata et al. used a previously published set of primer and probe of bcs31fragment in PCR- ELISA (Morata and Queipo-Ortuno, 2003). Some people have also used alkB/Is711 target gene based primers in both conventional (Doust and Ahmadi, 2007), and real-time PCR (Redkar and Rose, 2001). In an attempt to improve the sensitivity of a PCR assay for the detection of Brucella in clinical samples, we developed a DIG-ELISA using our own designed primer set. Primary PCR by N1/N2 primer pairs yielded PCR products between of 428bp. Using Brucella standard strains DNA as template for PCR also yielded the same products. Despite developing advanced and semiautomatic techniques for processing blood cultures and availability of many serological tests for indirect detection, there still are important problems in diagnosis of brucellosis. Blood cultures lack required sensitivity, and serological tests are not specific enough in regions where brucellosis is quite endemic (Young, 1991). Many patients with brucellosis also reported nonspecific symptoms after treatment and it is often difficult to decide whether the disease in the patient is following a favorable course, or relapsing the case has been take place(Ariza and Corredoira, 1995). Previous studies have demonstrated that the amplification of specific sequences of brucella genome is a much more sensitive method than blood culture and also is very specific during active infection (Morata and Queipo-Ortuño, 1999). However, most PCR procedures followed by electrophoresis with agarose gel and dot blot or southern hybridization. In order to improve the detection limit of conventional PCR, we first designed a new primer set and optimized it with standard brucella genome, and then developed and optimized a simple hybridization based microliter plate enzyme immunoassay for detection of amplified brucella DNA. The precision of PCR- ELISA can be considered good at least when tested with standard brucella and few clinical isolates. Bearing in mind that founding the inoculm in patients with brucellosis is very small; the detection capacity of a PCR technique should be high enough. The previous studies showed that conventional PCR is able to amplify 10 fg of bacterial DNA (Queipo-Ortuño and Morata, 1997). Nevertheless, dot blotting was required for the correct signal identification of the smallest amount of inoculum on agarose gel electrophoresis. Furthermore, in clinical samples, the high concentration of human DNA in peripheral blood samples occasionally interferes with the desired amplification (Morata and Queipo-Ortuño, 1998). Our evaluation of analytical sensitivity of the PCR-ELISA technique showed the ability to amplify 600 fg of bacterial

DNA. other researchers, using similar methods, have reported the detection limit of PCR-ELISA to be much more than present study (Morata and Queipo-Ortuno, 2003). Some people also reported the detection capacity of PCR-ELISA to be similar to that found with the ordinary PCR followed by southern hybridization (Jantos and Roggendorf, 1998). Sample size is a major limitation for PCR-based assays; the use of very small samples from patients with small concentrations of circulating brucella DNA could resulting the absence of amplifiable target DNA within the sample (Gamazo and Vitas, 1993). Although this study was not designed to analyze the usefulness of the PCR- ELISA for testing of clinical samples or posttreatment follow-up, the detection of 14 clinical isolates of brucella genomic DNA, confirm the reports about the effectiveness of this technique while dealing with clinical samples (Morata and Queipo-Ortuño, 1998; Morata and Queipo-Ortuño, 2003). The present results with the N1/N2 primers, demonstrated the high specificity of the technique with a wide panel of microorganisms. Only the DNA of Ochrobacterium species was recognized by these primer set (Khoramabadi and Doust, 2009). As well as the high sensitivity and specificity of this PCR- ELISA, there is also the advantage that with this procedure the detection of PCR products is rapid, easy, and objective; it requires no electrophoresis apparatus, UV light, or darkroom, and furthermore the use of toxic chemicals such as ethidium bromide. The technique also allows the simultaneous handling of a large number of samples and can be automated, making it attractive for use in clinical laboratories (Venturoli and Zerbini, 1998). Brucella species are class III pathogens requiring special protection measures; PCR-based assays almost completely obviate the need for direct handling of the pathogen, reducing the risk of laboratory personal contamination. In conclusion, the high sensitivity and specificity of this PCR-ELISA, together with the speed, versatility in sample handling, and laboratory risk reduction, make this technique quite useful tool for diagnosis of brucellosis both in human and livestock.

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