

Full Length Research Paper

Molecular detection of *Brucella* spp. from broth culture of clinical samples in Nigeria: Its role in vaccine quality control

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PCR was employed to detect *Brucella* spp. from broth cultures of clinical samples using a group specific primer based on IS6501 sequence. The sensitivity and specificity of this assay was confirmed by Southern hybridization analysis using a digoxigenin-labeled DNA probe while reproducibility of the analysis was confirmed by repetition of the test. Also, ERI₁ and ERI₂ primers were used to differentiate *Brucella abortus* strain 19 from other strains and the relevance in quality control of *Brucella* vaccine production highlighted.

Key words: PCR, Southern hybridization, *Brucella*, vaccine, quality control.

INTRODUCTION

Brucellosis is a zoonotic disease caused by gram-negative bacteria of genus *Brucella*. Based on host specificity, six species have been recognized; *Brucella abortus* (cattle), *B. canis* (dog), *B. melitensis* (Goat), *B. neotomae* (desert wood rats), *B. ovis* (sheep) and *B. suis* (pig, reindeer and hare (Meyer and Morgan, 1973; Morgan and Corbel, 1976). Several reports of *Brucella* species isolated from marine mammals, predominantly, seals and cetaceans have been made (Bricker et al., 2000). *Brucella* genus is highly homogenous with all members showing greater than 95% homology in DNA-DNA pairing studies, thus classifying *Brucella* as a monospecific genus (Verger et al., 1985).

Clinical signs of animal *brucellosis* vary from one animal species to the other. They include: abortion, retained placenta, orchitis, epididymitis, sterility, septicaemia, hygromas, non-suppurative synovitis, chronic bursal enlargement of neck and withers. Grossly, pathological lesions observed include necrotizing placentitis, disseminated inflammatory granuloma in aborted foetal tissues brachio or interstitial pneumonias (Radostits et

al., 1994).

In Nigeria, information on the epidemiology of *brucellosis* suggests that the disease is endemic with varying prevalence rates (Halle and Ajogi, 1997). The herding of cattle, sheep and goat provide conditions, which greatly increase the ease with which infections are transmitted from one animal group to another (Ocholi et al., 1993). Thus, there is a need for an accurate diagnosis for effective control program of brucellosis in Nigeria.

Microbiological isolation and identification are reliable methods of diagnosis for *Brucella* but are cumbersome and present great risk of infection for laboratory workers (Lopez-Merino et al., 1991). The work of Diaz-Aparicio et al. (1994), Gondswaad et al. (1976), have shown that serological methods of diagnosis are not always sensitive or specific because of cross reactions with related pathogens that often occur.

PCR is known to be of advantage in detecting DNA in pathogenic organisms that have been rendered biologically safe thus reducing the risk of infection of laboratory workers. Leal-Klevezas et al. (1995) have demonstrated the superiority of PCR technique over classical methods of diagnosis such as culture and serology, in its ability to detect small amount of *Brucella*.

Several molecular methodologies have been employed in the diagnosis of *brucellosis*: Bricker and Halling (1994),

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employed PCR assay in the differentiation of *B. abortus* bv.1, 2 and 4, *B. melitensis* and *B. suis* bv. 1 using five oligonucleotide primers, which can identify selected biovars. Also, single step PCR was used for detection of *Brucella* Spp from blood and milk of infected animals using primers based on the gene encoding for an external membrane protein (Omp-2) (Leal-Klevezas et al., 1995).

This study demonstrates the ability to detect *Brucella* DNA first from broth culture of clinical samples using PCR analysis and confirmed by Southern Hybridization.

MATERIALS AND METHODS

Source of Genomic DNA

The *Brucella* cultures were made available by the Bacteriology Research Department of the National Veterinary Research Institute, Vom, Nigeria. Broth cultures of the organism were made from clinical samples grown on serum dextrose agar.

Controls

The positive control was standard *brucella* DNA obtained from Applied Biotechnology (onderstepoort) Laboratory, South Africa while the negative control was a broth culture of a suspected case of *brucellosis* proven negative microbiologically and by PCR.

DNA preparation

DNA was extracted from broth cultures of *Brucella* using a modified method of extraction of *Brucella* DNA according to Romero et al. (1995). 500 ml of the broth culture was aliquoted into a micro centrifuge tube; 500 µl of buffer 1 (20 mM NaCl, 20 mM EDTA, 20 mM Tris-HCl pH = 7.5, 0.5% Triton x - 100) was added and left on ice for 30 min. The mixture was then centrifuged at 12000 rpm for 15 min at room temperature and the supernatant was discarded. The pellets were washed by adding 500 µl of 1x saline sodium citrate and vortexed and then centrifuged at 12000 rpm for 15 min and supernatant discarded. 500 µl of buffer 2 (10 mM NaCl, 50 mM EDTA, 50 mM Tris-HCl pH = 7.5, 1% sodium dodecyl sulphate) was added and vortexed, then 20 µl of proteinase k (20 mg/µl) was added and incubated at 50°C overnight to digest the cells. Digestion of cells was enhanced by boiling at 100°C for 5 min in a water bath (Precision: Scientific Inc.) 500 µl of biophenol (Phenol, chloroform and isoamyl alcohol (25:24:1)) then vortexed and centrifuged at 12000 rpm for 10 min.

The aqueous layer was transferred into a clean tube and 1/3 of the volume 3.5 M ammonium acetate and 2 volumes of absolute ethanol was added, mixed and centrifuged at 12000 rpm for 10 min to precipitate the DNA. The supernatant was discarded and the pellets (DNA) washed by adding 200 µl of 70% ethanol then centrifuged at 12000 rpm for 10 min. The supernatant was again discarded and DNA pellets dried in a heating block (BIBBY: Sterilin) at 56°C, and then dissolved in 30 µl of nuclease free water (Promega ®) and stored at -20°C.

Primers

The Primers used were *Brucella* group specific design for *Brucella* genus and they amplify the 650 bp fragment of the genome. They are:

Forward (ISP₁): 5' – GGT TGT TAA AGG AGA GC - 3'.
Reverse (ISP₂): 5' – GAC GAT AGC GTT TCA ACT TG-3'.

These were designed based on the IS6501 sequence of *Brucella* by Ouahrani-Bethach et al. (1996) and obtained courtesy of the Applied Biotechnology (Onderstepoort) Laboratory, South Africa.

ERI₁ and ERI₂ primers designed by Bricker and Halling (1995) amplifies 178bp fragment of all *B. abortus* strains except strain 19. They are based on the *eri* locus, which is associated with the ability to catabolize erythritol (sangari et al., 1994). The sequences are as follows:

Forward (ERI₁): 5'-GCG CCG CGA AGA ACT TAT CAA -3'.
Reverse (ERI₂): 5'-CGC CAT GTT AGC GGC GGT GA - 3'.

Primers IS711 and AB amplifies the 500 bp fragment and are *B. abortus* specific Bricker and Halling (1994). The sequences are as follows:

Forward (IS711): 5'-TGC CGA TCA CTT AAG GGC CTT CAT-3' (IS711-Specific Primers).
Reverse (AB): 5'-GAC GAA CGG AAT TTT TCC AAT CCC-3' (*B. abortus* –Specific Primers).

PCR

The amplification reactions using the above mentioned set of primers was prepared in volume of 21.0 µl (1x; 10 mM Tris-HCl pH 8.8 at 25°C, 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton x-100), nuclease free water (Promega ®), 2.50 µl Dynazyme buffer (Promega ®), 1.50 µl (37.5 mM) MgCl₂ (QIAGEN), 0.50 µl (5 mM) deoxynucleotide triphosphate (dNTP) (Promega ®), 0.25 µl (5 U/µl) Taq Polymerase (Roche ®). 4.0 µl of the template DNA was added to each reaction mixture. To reduce evaporation, the mixture was overlaid with 5 µl of mineral oil (Promega ®). The reaction was performed in a DNA thermal cycler (cyclogene: Techne (Cambridge) LTD, UK) for 30 cycles of 95°C for 35 s, 50°C for 45 s, 72°C for 45s.

Electrophoresis

Eight microlitre of the amplicons were fractionated in 1.5% Agarose (sigma®) gel using 1 xTBE (Tris-Borate-EDTA) (Promega ®) buffer to which 5 µl of ethidium bromide (1 µg/ul) (Promega ®) was added and then run at 60 volts for 60 min. The result were visualized under UV-light (sigma®) and recorded by photography using a MP-4 Land Polaroid camera (sigma®).

Southern hybridization

Southern hybridization analysis was used as a confirmatory technique for the PCR. Briefly, nylon membrane (Sigma ®), 3 mm filter paper (Whatman : England) and paper towels (sigma®) were cut to size (4 x 6 inches) and the edges and that of the gel were cut at the top-left corner for easy, identification. The gel was incubated in 0.25 M HCl for 15 min and then washed briefly with sterile distilled water and incubated with 20xSSC for a few minutes. Downward capillary transfer was then done overnight according to Koetsier et al. (1993).

The gel was later stained with ethidium bromide (5 µl) (Promega ®) and visualized under UV-light to confirm complete transfer of DNA to the nylon membrane. The nylon membrane was washed in 2-x SSC, air-dried and stored at 4°C between two 3 mm filter papers.

A digoxigenin labeled probe was prepared using a re-amplified amplicon of *Brucella* strain S19 DNA purified using Agarose Gel

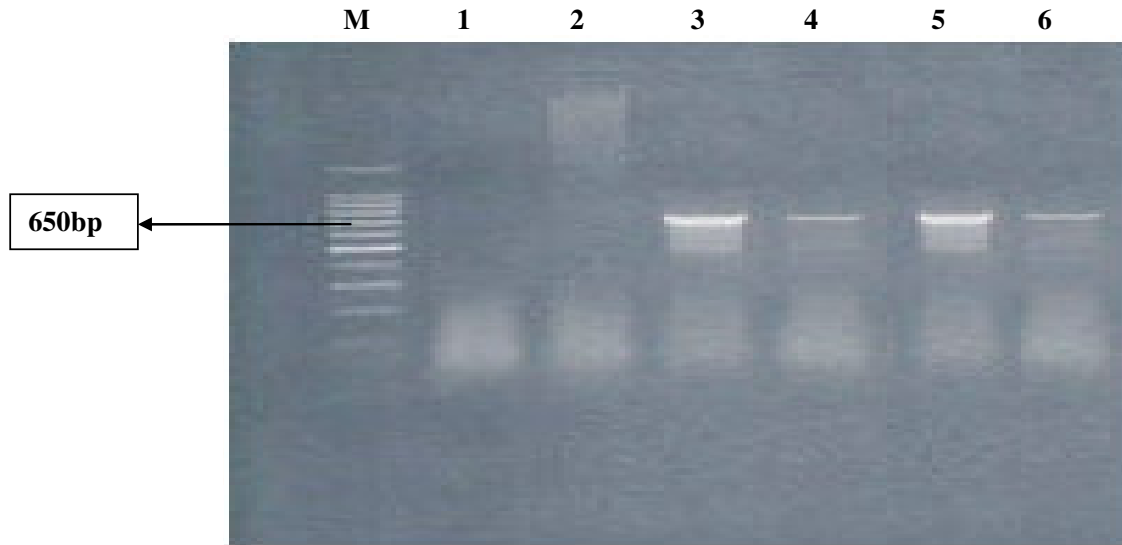


Figure 1. PCR result after electrophoresis of *Brucella* cultures. Lane M, 100 bp molecular weight marker; lane 1, control (water); lane 2, negative control; lane 3, positive control; lanes 4 – 6, *Brucella* cultures (all positive).

DNA Extraction Kit (Roche®). The purified DNA was washed by adding 2 volumes of absolute ethanol and kept at -20°C overnight then centrifuged at 14000 rpm for 15 min and supernatant discarded. This was repeated using 70% ethanol and then air-dried and 30 μl of nuclease free water was added.

Labelling and testing of the Probe (DNA) was done using High Prime DN Labelling and Detection Starter Kit 1 (Roche®) following the manufacturer's instruction.

Prehybridization and hybridization reaction was carried out on the above mentioned nylon membrane followed by stringency washes and then immunological detection, all according to manufacturer's instruction on the DIG High Prime DNA Labelling and Detection Starter Kit 1.

RESULTS AND DISCUSSION

Detection to the species level was not effected as the primers (1SP₁ and 1SP₂) are group specific and therefore will only amplify DNA sequences common to the *Brucella* genus. Going by the result of this study as shown in (Figure 1), the *Brucella* DNA (group specific) was amplified while non-*Brucella* DNA was not amplified using the above mentioned primers which are specifically designed for amplification of DNA of *Brucella* genus. It is note worthy that *Brucella* was once considered to be related to the genera *Bordetella* and *Alcaligenes* (Johnson and Sneath, 1973).

Ouahrani et al. (1996), demonstrated that the IS6501 (1SP₁ and 1SP₂) primers have around 10 times sensitivity compared to conventional method of PCR employed by Fekete et al. (1990). Thus, the effect of contamination by foreign DNA is insignificant as they will not be amplified especially at 650 bp fragment, ruling out the problem created by cross reactions related pathogen with *Brucella* using serological methods.

As a confirmatory test Southern Hybridization analysis

indicated bands formed as a result of hybridization of *Brucella* samples (DNA) to the probe (a digoxigenin – labelled known positive *Brucella* DNA). Moreover, the negative control showed no band, as there was no hybridization to the probe (Figure 2).

Lanes 1, 2 and 6 revealed a negative result as there were no observable bands formed as a result of hybridization indicating that the PCR technique used is specific and will only detect the DNA of *Brucella*. The result of the Southern hybridization analysis is significant as cross hybridization by contaminant which sometimes occur in Southern hybridization analysis thereby making interpretation of result difficult (Van kuppeveld et al., 1994) was overcome by the use of purified PCR product (*Brucella* DNA) for probe making to enhance sensitivity and specificity. Figure 2b shows the agarose gel electrophoresis of PCR product employed in the Southern hybridization test which serves as a confirmatory evidence of PCR reaction specificity.

In addition, we were able to differentiate by exclusion, *B. abortus* strain 19 from other strains of *B. abortus* using the primers ERI₁ and ERI₂ which amplifies a 178 bp fragment which represent an intact *eri* sequence in all *B. abortus* strain except strain 19 (Bricker and Halling, 1995). Samples were considered as abortus strain 19 if a 500 bp fragment was amplified with the IS7119 and AB primers and not by ERI₁ and ERI₂ set of primers (Figure 3). This may be of importance in the identification and/or confirmatory analysis of quality control program of *Brucella* vaccine production where *B. abortus* strain 19 is used.

Brucella vaccine production in Nigeria involves the use of *B. abortus* strain 19. Thus, the exclusion of other *B. abortus* strain which could act as contaminant during vaccine production process and the confirmation of the

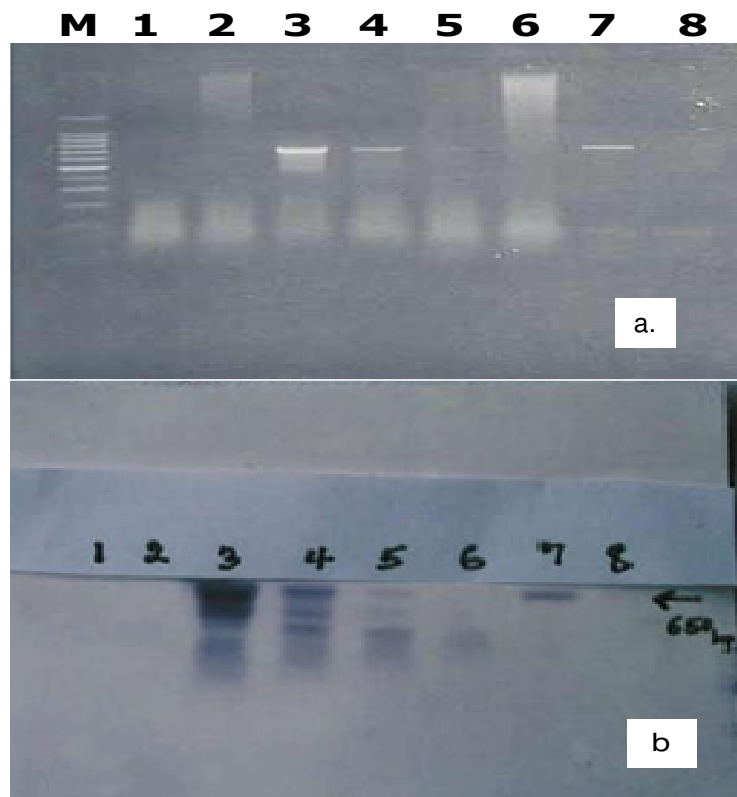


Figure 2. a. Agarose gel electrophoresis of PCR product employed in Southern hybridization. The sensitivity of this test was confirmed by Southern hybridization Figure 2b. All PCR analysis was performed under the same condition. b. Southern hybridization of *Brucella* cultures. Lanes 3, 4 and 5 show bands as a result of positive hybridization reaction, while lanes 1, 2 and 6 show no bands indicating a negative result. Lane 7 is positive control while lane 8 is the negative control.

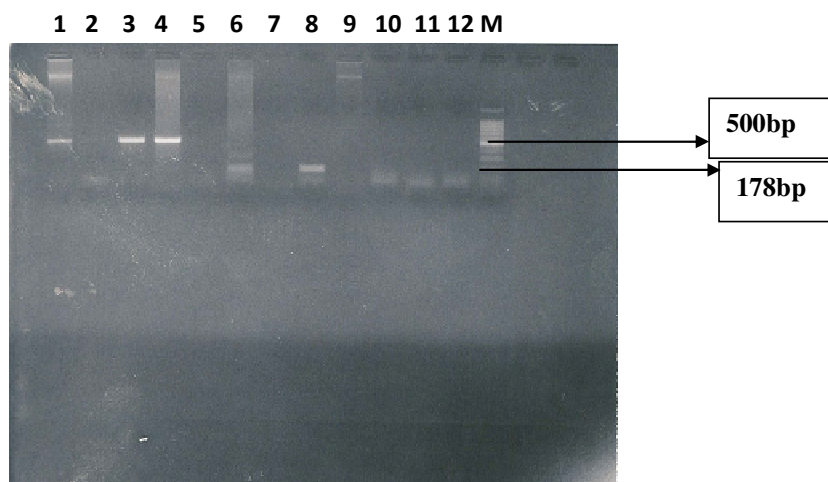


Figure 3. Agarose gel electrophoresis for primer based identification of *B. abortus* strain 19. Lane 1 (commercial brucellosis vaccine (*B. abortus* strain 19), and lanes 3 and 4 (*Brucella* broth cultures) show amplification of 500 bp fragment of *Brucella* genome (using *B. abortus* specific primers IS711 and AB). Lane 2 (same commercial brucellosis vaccine in lane 1), and lanes 5, 7, 9 – 12 (*Brucella* broth cultures) show no amplification, indicating *B. abortus* strain 19 (using ERI₁ and ERI₂). *B. abortus* strain 19 lacks the *eri* locus (a specific 178 bp fragment). Lanes 6 and 8 (same *Brucella* broth cultures in lane 3 and 4) show amplification of 178 bp fragment indicating non-*B. abortus* strain 19 (using ERI₁ and ERI₂ primers). M: 100 bp molecular weight marker.

presence of the vaccine strain are of utmost importance in quality control procedure and quality assurance of *Brucella* vaccine produced. Furthermore, the control of *brucellosis* is among other factors, dependent on the effective vaccination of live-stock with quality assured vaccine.

All amplifications were repeated two times with same result indicating the reproducibility of this assay. Although, the need for PCR detection of *Brucella* DNA to the specie level and direct detection from clinical samples cannot be over emphasized this result indicates the specificity and sensitivity of our PCR analysis of *Brucella* genus using cultures of *Brucella* colonies isolated from infected samples.

Considering the current epidemiology of *brucellosis* in developing countries like Nigeria and the zoonotic importance of this disease, the use of PCR assay for accurate detection is paramount where specificity and sensitivity are not compromised.

Also, a successful fight against *brucellosis* and the role of *Brucella* in biological warfare and agro-terrorism as pointed out by Bricker (2002) makes molecular diagnosis a vital tool that must also be accessed by developing countries. Furthermore, a reliable diagnostic and quality control tool such as described in this work should be employed in order to ensure proper control of the disease in animal and human population.

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