POLYMERASE CHAIN REACTION FOR DETECTION OF *BRUCELLA* SPECIES: UTILITY IN DIAGNOSIS OF ANIMAL INFECTION

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ABSTRACT

A polymerase chain reaction (PCR) assay was used to amplify a 216-base pair (bp) sequence of *Brucella* DNA using primers specific for the gene encoding for a 31-kDa protein. Genomic DNA from all of 3 *Brucella abortus* and *Brucella melitensis* reference strains and 4 *Brucella* local isolates were analyzed and yielded exclusively the 216-bp amplified target fragment. No amplification was detected with DNAs from 2 gram-negative bacteria showing serological cross-reaction with *Brucella* species. The sensitivity of PCR detection was determined using different concentrations of genomic *Brucella abortus* and *Brucella melitensis* reference strain DNA. As little as 100 fg of *Brucella* DNA was detected by this method. To evaluate the diagnostic ability of the assay for the detection of *Brucella* species in field samples, a total of 52 bovine fetal and maternal samples were tested by PCR and bacteriological examination. Of 28 bacteriologically -positive samples, 27 were positive by PCR. Of 24 culture-negative samples, 1 gave a positive result by PCR. The PCR showed a sensitivity of 96.4% and specificity of 95.8% when compared with bacteriological examination. The specificity and high sensitivity of PCR assay and its ability to detect *Brucella* species in field samples may provide a valuable tool for the rapid diagnosis of animal brucellosis.

INTRODUCTION

Brucellosis is a widespread and economically important zoonosis, which is also infectious for humans (World Health Organization, 1986). It is caused by gram-negative bacteria of genus *Brucella*. Brucellosis of bovine and small ruminants caused, respectively, by *Brucella abortus* and *Brucella melitensis* frequently results in a marked decrease in reproductive efficiency owing to abortion, clinical disease, infertility and diminished levels of milk production (Crawford et al., 1990).

The diagnosis of brucellosis is currently based on serological and microbiological tests. It is known that serological methods are not always sensitive or specific (Diaz-Aparicio et al., 1994; Perry and Bundle, 1990). Moreover, they have been reported to cross-react with antigens other than those from *Brucella* species (Alton et al., 1988). Microbiological isolation and identification are the most reliable methods of diagnosing brucellosis. However, these procedures are time-consuming and lack sensitivity for individuals with chronic infections (Lulu et al., 1988), and represent great risk of infection for laboratory diagnosticians. Because of these difficulties, the development of new diagnostic testes for the direct detection of *Brucella* species in clinical samples is increasingly interesting.

PCR assay has been shown to be a valuable method to detect DNA from different microorganisms. Although there are several studies on *Brucella* DNA detection by PCR with pure cultures (Bialy et al., 1992; Fekete et al., 1990; Herman and De Ridder, 1992; Romero et al., 1995; Tcherneva et a1., 1996), few studies have been performed with clinical or field samples (Fekete et al., 1992; Gallien et al., 1998). This study was planned to apply the PCR assay as a rapid and sensitive method for the detection of *Brucella* species and to determine its utility in the diagnosis of animal brucellosis.

MATERIALS AND METHODS

Bacterial strains.

Brucella reference strains and local *Brucella* field isolates used in the present study are listed in Table 1. Local field isolates were isolated, identified and typed by colonial morphology, Gram staining, biochemical and antigenic characteristics according to the procedures described by Alton et al., (1988). *Escherichia coli* O: 111 and *Yersinia enterocolitica* O: 9 were used as negative controls and selected because of their reported cross-reactivity with *Brucella* species in serological tests. The *Brucella* strains and *Brucella* isolates were cultured and tested morphologically, culturally, biochemically and antigenically to be sure that they are *Brucella* species as previously described (Halling and Zehr, 1990). Culture conditions were used for the other organisms as appropriate (Ibrahim et al., 1992; Woodward et al., 1992).

Extraction of genomic DNA.

DNA was isolated as described by Fekete et al. (1992). Briefly, bacterial cells from 10 ml of culture were collected by centrifugation at 10,000 Xg for 15 minutes at 4°C. After washing in phosphate buffer saline, the cellular pellets were resuspended in lysis buffer (10 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl [pH 7.6]), Ribonuclease A was added to a final concentration of 50 µg/ml and the mixture was incubated for 1 hour at 50°C. Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5% and Protinase K was added to a final concentration of 500 µg/ml. The reaction mixture was incubated for 2 hours at 50°C and was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was extracted with chloroform-isoamyl alcohol (24:1), from which the aqueous phase was retained. DNA was precipitated with 100% cold ethanol after addition of sodium acetate to a final concentration of 0.3 M and collected by centrifugation. After washing with 70% ethanol, the DNA pellet was dried before being resuspended in 25 µl of sterile ultrapure water. The DNA concentration was measured by spectrophotometry at 260 nm. DNA preparations were stored at -20°C until further use.

PCR target selection and primers.

Two 17-mer primers B1: 5' TCG GTT GCC AAT ATC AA 3' and B2: 5' CTT GCC TTT CAG GTC TG 3' were used for amplification of *Brucella* DNA (Baily et al., 1992). The primers were synthesized at the Nucleic Acid Facility at Iowa State University, Ames, IA, USA. The 216-bp target is located between bases 723 and 1008 of the gene encoding for a 31 KDa *Brucella* protein and selected from region of high conservation of nucleotide and amino acid sequence (Mayfield et al., 1988).

PCR.

Amplification reaction mixtures were prepared in a volume of 20 µl containing 50 mM KCl, 10 m M Tris-HCl (pH 8.3), 200 µM (each) deoxynucleotide triphosphate (dNTP), 500 nM each primer, 1.5 mM $MgCl_2$, 0.5 U Taq DNA polymerase (Advanced Biotechnologies, Surrey, UK) and 10 ng of genomic DNA. A layer of 20 µl of mineral oil was added to prevent evaporation of the reaction reagents. The amplification reaction was performed in a DNA thermal cycler (Model 110 P, Coy Corp., Grass Lake, MI, USA). After initial denaturation at 90°C for 5 minutes, the PCR profile was set as follows: 1 minute of each template denaturation at 90°C, primer annealing at 60°C, primer extension at 72°C, for a total of 40 cycles, with a final extension at 72°C for 10 minutes. Negative controls containing all of the reagents but lacking template DNA were processed exactly as described above to monitor carry- over contamination with *Brucella* DNA. Each sample was tested twice in different independent experiments.

Agarose gel electrophoresis of PCR products.

After amplification, 7 µl of the amplification reaction mixture was taken for electrophoresis at 90 volts for 45 minutes on 1% agarose gel containing 1X Tris-Acetate-EDTA buffer (Sambrook et al., 1989), stained with ethidium bromide (0.5 g/ml), observed under UV light and photographed with type 667 Polaroid film. The presence of a clear -cut DNA band of 216 bp, compared to the molecular weight size marker, was regarded as a positive result.

Sensitivity of *Brucella* detection by PCR.

The sensitivity of PCR assay was determined by using purified genomic DNA from *Brucella abortus* 544 and *Brucella melitensis* 16 M reference strains. DNA stock from each strain was serially tenfold diluted in sterile ultrapure water (from 1 ng to 10 fg) and aliquots of 1µl from each dilution were analyzed by PCR as described above.

Processing, PCR testing and bacteriological examination of field samples.

A total of 52 bovine fetal and maternal field samples from different organs (fetal stomach contents and maternal uterine exudates, placental cotyledons and supramammary lymph nodes) were collected from serologically positive bovines by standard rose bengal and tube agglutination tests for brucellosis. The samples were sent to ARRI be tested for *Brucella* species. Samples to be used for PCR testing were stored at -20°C, while those used for bacteriological assay were kept at 4°C until being tested within 24 hours. Frozen tissue samples (about 100 gm) were allowed to thaw at room temperature, cut into small pieces and then homogenized in sterile PBS in plastic bags before being subjected for DNA extraction. Samples from fetal stomach content and uterine exudate were used directly for DNA extraction. PCR amplification was performed as described above for bacterial strains. Culture-negative placental tissue samples from *Brucella*-free cattle were included as negative controls. For bacteriological testing, tissue homogenates or fluid samples were streaked on plates of brucella agar selective media. Seeded plates were incubated in 10% CO_2 atmosphere for at least 3 days. Suspected *Brucella* colonies were picked up, purified and identified according to the standard procedures described by Alton et al., (1988). The sensitivity and specificity of the PCR assay as a diagnostic test were calculated as described for the analysis of testing individual animals (Monke et al., 1992). To define sensitivity and specificity,

results of the PCR were compared with those of bacteriological examination. The sensitivity of the PCR assay was calculated by dividing the number of samples that tested positive by PCR by the number of samples found positive by culture. The specificity of the PCR assay was calculated by dividing the number of samples that tested negative by PCR by the number of samples found negative by culture.

RESULTS

PCR AMPLIFICATION OF DNA FROM *BRUCELLA* REFERENCE STRAINS AND LOCAL ISOLATES

The results from the PCR amplification of *Brucella* DNA from reference strains and local isolates using the B1 and B2 primers are given in fig.1. As predicted, one band of the expected size (216 bp) was amplified from the DNAs of *Brucella abortus* 544, *Brucella abortus* S19 and *Brucella melitensis* 16M reference strains (Fig.1A). Similarly, DNAs from local *Brucella abortus* biovar 1 and *Brucella melitensis* biovar 3 tested isolates yielded identical amplified DNA bands of the same molecular weight on agarose gel (Fig. 1B). On the other hand, no amplification was detected for DNAs from *Escherichia coli* O: 111 and *Yersinia enterocolitica* O: 9 (lanes 6 and 7 respectively).

SENSITIVITY OF *BRUCELLA* DETECTION BY PCR

To determine the sensitivity of *Brucella* DNA detection by PCR amplification, a 10 fold dilution series of *Brucella abortus* 544 and *Brucella melitensis* 16M reference strain purified genomic DNA was subjected by PCR. Fig.2 shows the results of agarose gel electrophoresis of PCR products following amplification of different *Brucella* genomic DNA dilutions tested. As little as 100 fg of genomic *Brucella* DNA was detected and visualized on agarose gel (panels A and B, lane 7). No detectable difference was observed between *Brucella abortus* and *Brucella melitensis* regarding the detection limit by PCR amplification.

PCR TESTING AND BACTERIOLOGICAL EXAMINATION OF FIELD SAMPLES

To assess the utility of the PCR assay in the detection of *Brucella* DNA in field samples from infected animals, a total of 52 bovine fetal and maternal samples were tested by PCR and culture procedures. As shown in Fig.3, *Brucella* DNA was detected by PCR amplification in samples taken from fetal stomach contents, uterine exudates, placental cotyledons and supramammary lymph nodes, demonstrated by the presence of a single DNA fragment of 216 bp. No amplification was observed for DNA from culture-negative placental tissue samples from *Brucella*-free cattle used as negative control (lane 6). A two-by-two comparison of the results with PCR assay and culture procedures are shown in table 2. Of 28 culture-positive samples, 27 were positive by PCR. Of 24 culture-negative samples, 1 gave a positive result by PCR. The sensitivity of the PCR assay was 96.4% compared with culture techniques and its specificity was 95.8%.

DISCUSSION

The fight against brucellosis in animals relies mainly on veterinary sanitation measures focused on the reduction or eradication of this disease in farm animals. A critical tool to the success of these measures is unquestionably a rapid and accurate

diagnosis of the disease. Therefore, the aim of this study was to use of the PCR assay for detection of *Brucella* species and to assess its utility in the diagnosis of brucellosis in animals.

In the present study, a PCR assay was used to amplify *Brucella*-specific target DNA sequences. The oligonucleotide primer set used for amplification was specific for the gene encoding for a 31 KDa *Brucella* outer membrane protein, a region of high nucleotide and amino acid conservation among *Brucella* species. Purified genomic DNAs (10 ng/ 20-µl assay mixture) from each of the *Brucella* reference strains and local isolates were evaluated as targets. DNA was amplified from 2 *Brucella* species including *Brucella abortus* biovar 1 and *Brucella melitensis* biovar 1 and 3. The lack of amplification when genomic DNAs extracted from *Escherchia coli* O: 111 and *Yersinia enterocolitica* O: 9 that are repeatedly reported to cross-react serologically with *Brucella* species (Alton et al., 1988) support the specificity of PCR amplification of *Brucella* sequences.

In this study, a DNA – free test sample was included in each run of PCR amplification as a control for contamination by *Brucella* target DNA or PCR product. No carryover contamination by target DNA or amplified product was observed (represented in fig.2, lane 2).

In this study, a sensitivity test was conducted to ascertain the limit of detection of the PCR assay. Decreasing concentrations of purified genomic *Brucella* reference strain DNA were subjected to PCR amplification. There was no difference in the threshold sensitivities of the PCR for *Brucella abortus* 544 and *Brucella melitensis* 16M tested (100 fg of DNA). Similar limits of detection were reported by Romero et al. (1995).

In order to assess the utility of the PCR test, a variety of bovine fetal and maternal field samples were evaluated for the detection of *Brucella* species. PCR assay with DNAs from bovine different fluid or tissue field samples yielded the same amplified fragment of 216 bp, as the ones from *Brucella* reference strains and local isolates, whereas DNAs extracted from culture-negative placental tissue samples failed to serve as template in the PCR. These results indicate the specificity of DNA extraction and PCR protocol for the selective detection of *Brucella* species in samples from infected animals.

PCR sensitivity and specificity of 52 field samples were compared with bacteriological testing. When the PCR was applied to the field samples, its sensitivity with respect to bacterial culture was 96.4% (of 28 culture-positive samples, 27 were PCR-positive). The presence of polymerase inhibitors (Rolfs et al., 1992) could account for a PCR-negative result in the sample that was culture-positive. Many substances has been suggested to be amplification inhibitors; including hemoglobin, urine, heparin, phenol and SDS (Gelfand and White, 1990; Jackson et al., 1992). Other factors that may account for the false-negative PCR result are a number of *Brucella* organisms below the detection limit, the degradation of target DNA in the sample, and inefficient DNA extraction. The PCR assay showed a specificity of 95.8% (of 24 culture-negative samples; only 1 gave a positive result by PCR). A false-negative bacteriological result can be caused by

massive contamination of the samples, by inhibition of some *Brucella* species in the selective media (Blasco, 1992), or by a viability loss before culturing, and in all these circumstances DNA can still be detected by PCR.

Beside its sensitivity and specificity, the PCR assay described in this study has several advantages over the current microbiological diagnostic methods for *Brucella* species. A major advantage is the speed with which the assay can be performed, where results could be obtained within less than 24 hours. Conventional methods require at least several days or even weeks. Moreover, it eliminates the hazards of handling the organism in the laboratory. This is significant because *Brucella* is a human pathogen.

The results presented in the present study have demonstrated the reliability, high sensitivity and specificity of the PCR assay for use in the detection of *Brucella* DNA and its utility in detecting the presence of the *Brucella* species in field samples from naturally infected animals. These data may form a strong basis for the development of new alternative diagnostic methods for brucellosis in animals.

It is clearly important not only to detect but also to identify the species of *Brucella* implicated in natural infection. Development of PCR - based methods that can be used to easily differentiate between *Brucella* species and biovars needs further studies. This would be of significance for studies on the epidemiology of brucellosis in animal livestock.

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Table 1: *Brucella* strains and isolates used in this study.

Species and biovars	Strain	Source	Origin
B. abortus 1	544	Reference	CVL*, Weybridge, UK
B. abortus 1	S19	Reference vaccine strain	CVL*, Weybridge, UK
B. abortus 1		Local Field Isolate (cow milk)	ARRI**, Giza, Egypt
B. abortus 1		Local Field Isolate (buffalo milk)	ARRI**, Giza, Egypt
B. abortus 1		Local Field Isolate (cow uterus)	ARRI**, Giza, Egypt
B. melitensis 1	16M		CVL*, Weybridge, UK
B. melitensis 3		Local Field Isolate (sheep lymph node)	ARRI**, Giza, Egypt

* CVL: Central Veterinary Laboratory
** ARRI: Animal Reproduction Research Institute

Table 2: Comparison of the results of PCR testing and culture assay with bovine field samples.

Test result	PCR-positive	PCR-negative	Total
Culture-positive	27*	1	28
Culture-negative	1	23**	24**
Total	28*	24	52

* Sensitivity of the PCR assay was calculated to be 96.4% compared to culture assay.
** Specificity of the PCR assay was calculated to be 95.8% compared to culture assay.

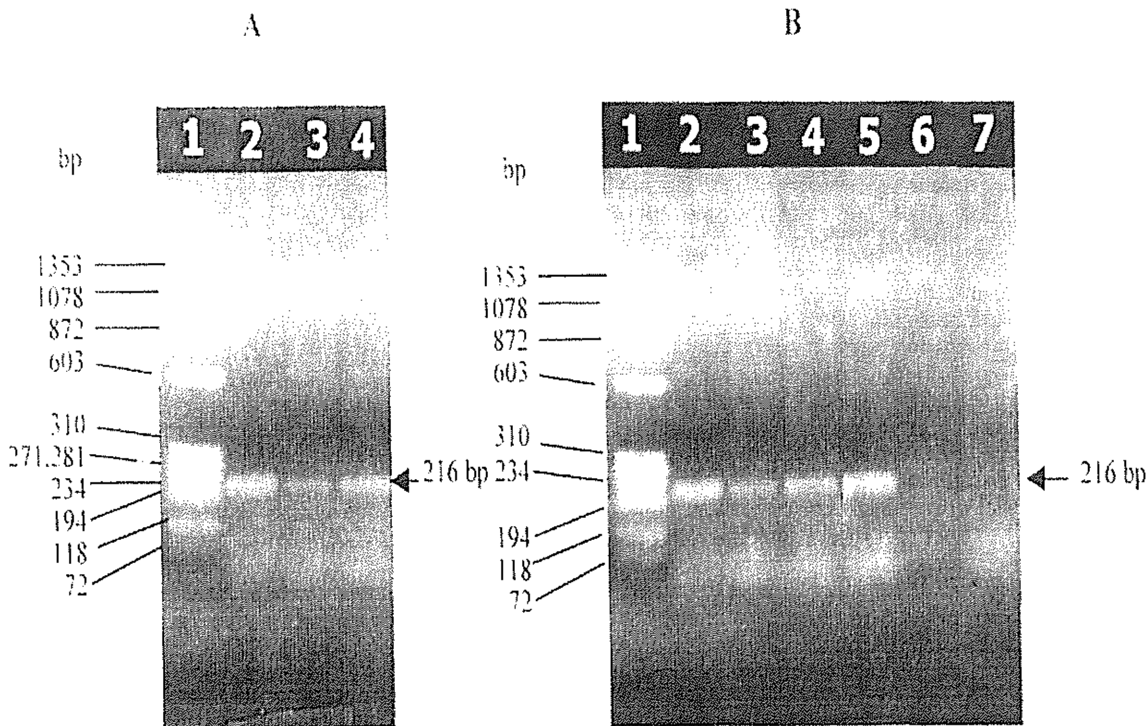

Fig. 1. PCR amplification products from *Brucella* strains (A) and local isolates (B) tested with the B1 and B2 primers. A 7-μl sample of each PCR product was resolved by electrophoresis in agarose, visualized by ethidium bromide and photographed. A) Lane 1: molecular weight marker (ΦX 174 Hae III DNA); Lane 2: *B. abortus* 544; Lane 3: *B. abortus* S19; Lane 4: *B. melitensis* 16M. B) Lane 1: molecular weight marker. Lanes 2-4: *B. abortus* biovar 1 isolates from cow milk, buffalo milk, and cow uterus respectively. Lane 5: *B. melitensis* biovar 3 isolate from sheep lymph node; Lane 6: *E. coli* O: 111; Lane 7: *Y.enterocolitica* O: 9. Numbers along the left margin of each panel indicate the sizes (in bp) of selected fragments of the molecular weight marker. An arrow on the right indicates the 216-bp amplified fragment.

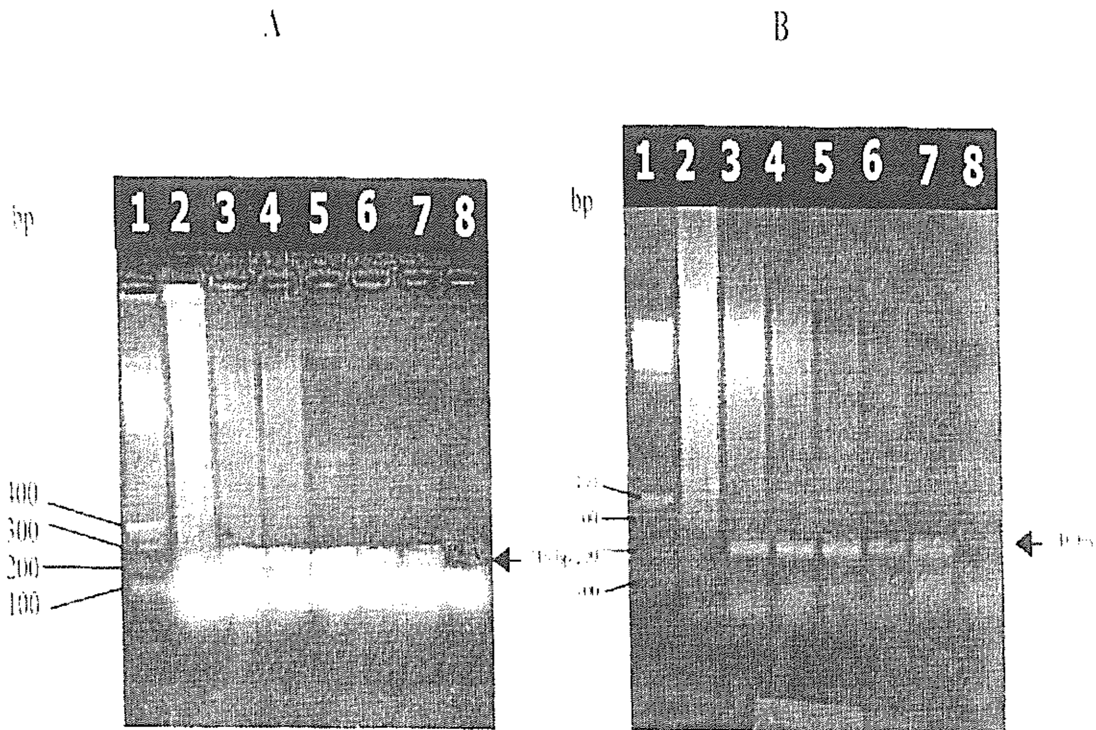

Fig. 2. Sensitivity of PCR amplification for the detection of DNA from *B. abortus* 544 (A) and *B. melitensis* 16M (B) reference strains. Tenfold serial dilutions (from 1 ng to 10 fg) of purified genomic DNA were analyzed by PCR. A 7-μl sample of each PCR product was resolved by electrophoresis in agarose, visualized by ethidium bromide and photographed. Lane 1: molecular weight marker (100 bp ladder); Lane 2: negative control reaction with no template DNA; Lanes 3-8: serial *Brucella* DNA concentrations used. Numbers along the left margin of each panel indicate the sizes (in bp) of selected fragments of the molecular weight marker. An arrow on the right indicates the 216-bp amplified fragment.

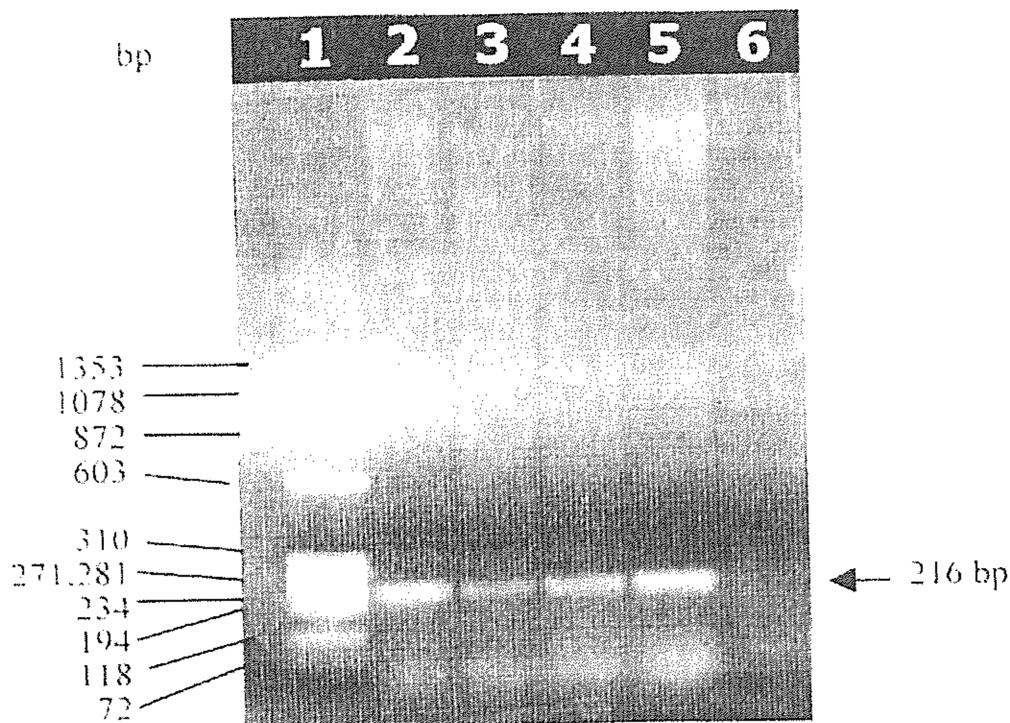

Fig. 3. Representative PCR amplification products from the tested bovine field samples. A 7-µl sample of each PCR product was resolved by electrophoresis in agarose, visualized by ethidium bromide and photographed. Lane 1: molecular weight marker (ΦX 174 Hae III DNA); Lane 2: fetal stomach content; Lane 3: uterine exudate; Lane 4: placental cotyledon; Lane 5: supramammary lymph node. Lane 6 represents placental tissue sample from *Brucella*-free cattle (negative control). Numbers along the left margin of each panel indicate the sizes (in bp) of selected fragments of the molecular weight marker. An arrow on the right indicates the 216-bp amplified fragment.

الملخص العربى

مدي فائدة استخدام اختبار سلسلة إنزيم البلمرة في تشخيص الإصابة الحيوانية بميكروب البر وسيلا

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استخدم اختبار تفاعل إنزيم البلمرة في مضاعفة تتابع نيكلوتيدي من ٢١٦ زوج من القواعد من الحمض النووي المميز لميكروب البروسيلا وذلك باستخدام زوج من بادئات التفاعل خاص بجين يترجم لبروتين وزنه الجزيئي ٣١ كيلو دالتون . تم اختبار ٣ عترات مرجعية من البر وسيلا ابورتس والبر وسيلا ميليتنسس بالإضافة إلي ٤ عترات بروسيلا معزولة محليا وأعطت جميعا نتائج إيجابية للكشف عن الحمض النووي الخاص بميكروب البروسيلا. ولم يعط الاختبار أي نتائج إيجابية بالنسبة لنوعين آخرين تم اختبارهما من الميكروبات سالبة الجرام والمعروفة بحدوث تفاعلات غير نوعية مع ميكروبات البروسيلا سيرولوجيا . وقد تم تحديد مدي حساسية الكشف بواسطة اختبار تفاعل سلسلة إنزيم البلمرة باستخدام تركيزات مختلفة من الحمض النووي الديوكسي ريبوزي المستخلص من عترات قياسية لكلا من البروسيلا ابورتس والبروسيلا ميليتنسس حيث تم الكشف عن حتى تركيز ١٠٠ فيكوجرام من الحمض النووي بواسطة هذا الاختبار .

ولتحديد القدرة (الكفاءة) التشخيصية لاختبار تفاعل سلسلة إنزيم البلمرة للكشف عن ميكروبات البر وسيلا في العينات الحقلية من الأبقار ، تم اختبار عدد ٥٢ عينة من العينات الجنينية والأبقار الأمهات بواسطة كل من هذا الاختبار والفحص البكتيريولوجي وقد دلت النتائج أن عدد ٢٧ من أصل ٢٨ عينة أعطت نتائج إيجابية بواسطة الفحص البكتيريولوجي كانت إيجابية بواسطة اختبار تفاعل سلسلة إنزيم البلمرة ، ومن بين ٢٤ عينة أعطت نتائج سالبة بالفحص البكتيربولوجي كانت عينة واحدة إيجابية لاختبار تفاعل سلسلة إنزيم البلمرة . وطبقا لهذه النتائج فقد تم حساب كل من حساسية وتخصص الاختبار بنسبة ٩٦,٤% ، ٩٥,٨% مقارنة بالفحص البكتيريولوجي علي الترتيب . وقد أمكن الاستنتاج نظرا لما يتميز به اختبار تفاعل سلسلة إنزيم البلمرة من الكشف بتخصص والحساسية الفائقة والقدرة علي الكشف عن الحمض النووي المميز للميكروب في العينات الحقلية فانه يمكن استخدام الاختبار كاختبار ذو فائدة قيمة في التشخيص السريع للإصابة بميكروب البروسيلا في الأبقار.