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Detection of *Mycobacterium avium* subspecies *paratuberculosis* using nested polymerase chain reaction (nPCR)

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis*, the cause of paratuberculosis (Johne's disease) is a chronic debilitating infection in ruminants. The disease is one of the most widespread bacterial infections of ruminants throughout the world. Recently, the organism was reported to be associated with enteric infection in humans and hence the disease is of public health importance. In the present study, a nested Polymerase Chain Reaction (nPCR) was developed and evaluated for detection of *Mycobacterium avium* subspecies *paratuberculosis*. The insertion sequence (IS) 1311 was used as a target DNA for the nested PCR amplification. First, a pair of primers (P1 and P2) was designed from IS1311 nucleotide sequences to produce 549 bp PCR products. Next, a second pair of internal (nested) primers (P3 and P4) was used to produce a 260 bp PCR product. The nested amplification was necessary to increase the sensitivity of the PCR assay and to confirm the identity (specificity) of the first amplified PCR product. Because it is a highly sensitive technique the nPCR could be used for detection of Johne's disease at an early stage of the infection. In addition, the described nPCR assay could be used as a supplement, or as an alternative, to conventional bacteriological procedures currently used for diagnosis of the disease in susceptible humans or animal populations.

Key words: *M. avium* subspecies *paratuberculosis*, Johne's disease, nPCR

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Introduction

Johne's disease (paratuberculosis) is a chronic debilitating infection of ruminants caused by *Mycobacterium paratuberculosis.* Recently, the name of the organism has been changed to *Mycobacterium avium* subspecies *paratuberculosis* (SIGURDARDOTTIR et al., 1999). The disease is common in cattle, but sheep and goats may also be susceptible to infection (ABBAS et al., 1986; ABUBAKER and ELSANOUSI, 1975). The economic importance of the disease is mainly attributed to reduced productivity, poor subsequent reproductive performance and culling of infected animals (BENDECTUS et al., 1987). The disease is distributed worldwide and Sudan is one of those countries that are exposed to the disease (FAWI and OBIED, 1964; ABUBAKER and ELSANOUSI, 1975; ABBAS et al., 1986; MUNGASH, 1989). The disease was diagnosed on the basis of clinical signs (FAWI and OBIED, 1964). However, the first isolation of the organism was reported by (ABBAS et al., 1986) from clinically infected goats. Tentative diagnosis of paratuberculosis could be made by demonstration of the acid-fast organism in stained slides. However, bacteriological examination would be necessary for definitive diagnosis of the disease. Despite the development of modern methods and techniques for isolation and diagnosis of Johne's disease, its control remains a major veterinary problem (MERKAL, 1984). One of the problems associated with the disease is that symptoms appeared lately and after a long time. (SIGURDARDOTTIR et al., 1999). In addition, isolation and identification procedure is time consuming, cumbersome and may take several months (MUNGASH, 1989). Serological techniques could also be used to identify a previous exposure to the disease but not an active infection. Nucleic acid hybridization techniques were applied successfully for the detection of the disease. Polymerase chain reaction (PCR) provides rapid, sensitive and specific detection for an early diagnosis of the disease (SAIKI et al., 1989). In the present study, a nested PCR was developed and evaluated for detection of *Mycobacterium avium* subspecies *paratuberculosis.*

Materials and methods

Strains of bacteria. Strains of *M. avium* subspecies *paratuberculosis* were isolated from intestines and lymph nodes of clinically infected goats

showing symptoms of emaciation, profuse diarrhoea and corrugation of the intestine. The bacterial isolates were provided by Dr. Zakia A of the Central Veterinary Research Laboratory, Khartoum, Sudan. The total nucleic acid extracts from *M. tuberculosis* and *M. avium*, closely related acid-fast bacteria, were provided by Dr. SARRA, A. (negative control) from the Central Veterinary Research Laboratory, Soba, Khartoum, Sudan. The isolates were purified by single colony picks three times. The biochemical characterizations of these isolates were based on conventional bacteriological methods (ABUBAKER and ELSANOUSI, 1975).

Bacterial growth and extraction of nucleic acid. The organisms were grown in Luen Stien Johnson's medium (Difco Laboratory, Detroit, MI) and incubated at 37 ^{0}C. Total genomic DNA was extracted from the bacterial isolates. In this study whole DNA was used without initial purification. Briefly, the cultures were centrifuged at 4,000 rpm in a bench centrifuge for 15 min to sediment the organisms. The pellet was resuspended in lysing buffer containing 300 microlitres (µl) of 0.1 M Tris pH 8.0; 50 µl of 10% sodium dodecyl sulphate (SDS). The mixture was incubated at 37 ^{0}C for 30 min and at 56 ^{0}C for 30 min. The mixture was then transferred to 1.5 ml Eppendorf tube and extracted with an equal volume of phenol/chloroform/ isoamylalcohol at a ratio of 25:24:1, respectively. The supernatant was transferred into a clean sterile Eppendorf tube and extracted with chloroform/isoamylalcohol (24:1). The addition of 2 volumes of absolute ethanol precipitated the extracted DNA in the aqueous phase. The extracted DNA was washed in absolute alcohol, vacuumed dried and dissolved in 50 µl of double distilled water (ddH_2O). Five µl of the resuspended DNA were used in the nPCR.

Primers design. Primers (20mer each) were selected from the published sequence of the insertion sequence (IS) 1311 of *M. paratuberculosis* and used in these PCR assays (WHITTINGTON et al., 1998). Primers 1 and 2 (P1 and P2) were selected for the synthesis of specific PCR product. P1 included bases 181-200 of the positive sense strand: (5)- TGAACGGAGCGCATCACGAA- (3). P2 included bases 711-730 of the complementary strand: (5)- TGCAGCTGGTGATCTCTGAT (3). The nPCR using primer P1 and P2 would result in a 549 bp product. For synthesis of a nested (internal) amplified product, internal to primers P1

and P2, oligonucleotide primers (P3 and P4) were selected from the published sequence cited above. P3 and P4 were internal to the annealing sites of P1 and P2. P3 consisted of bases 381-400 of the positive strand (5) GCAAAGTCGACGATTTGGTC (3). P4 was designed from the complementary strand between bases 621-640 (5) AAGTGCTCGGCTTCGACGTC- (3). PCR amplification using P3 and P4 would result in 260 bp PCR product internal to the annealing sites of P1 and P2. All primers were synthesized on a DNA synthesizer (Milliigen/ Biosearch, a division of MilliporeBurlington, MA, U.S.A.) and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, Virginia, U.S.A.)

PCR protocol for M. paratuberculosis detection. A stock buffered solution containing 250 microlitres (µl) 10X PCR buffer, 12.5 µl of each dATP, dTTP, dGTP and dCTP was prepared in a 1.5 ml Eppendorf tube. The primers were used at a concentration of 20 micromole (µM) per litre (L), and double distilled water was added to bring the volume of the stock buffer solution to 1.5 ml 5.0 µl of the target DNA was added to 44 µl of the stock solution in PCR tubes and mixed by vortexing. 1.0 µl of Taq DNA polymerase (Perkin Elmer) was used at a concentration of 5.0 units. A drop of mineral oil was used to cover the reaction mixture. All PCR amplification reactions were carried out in a final volume of 50 µl. The thermal cycling profiles were as follows: a 2-min incubation at 94 ^{0}C, followed by 30 cycles of 94 ^{0}C for 1 min, 55 ^{0}C for 30 sec and 72 ^{0}C for 45 sec. A final incubation at 72 ^{0}C for 10 min was carried out to ensure complete synthesis of the expected PCR products.

The nested amplification used in this study was described in detail in a protocol described by ARADAIB et al., (1998b). Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ.). Following amplification, 20 µl from each PCR reaction containing amplified product were loaded onto gels of 1.5% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and the PCR products were identified following visualization under UV light.

Results

The first amplification using the outer primers P1 and P2 afforded sensitive and specific detection of *M. avium* subspecies *paratuberculosis* strains used in this study. The specific 549 bp PCR product was visualized on ethidium bromide-stained gel from 1 nanogram (ng) DNA of bacteria isolated from naturally infected goats. The signal is faint at lower concentrations of DNA but is still visible. The amount of 1.0 ng DNA extracted from the *M. tuberculosis* and *M. avium* failed to demonstrate PCR products (Fig.1).

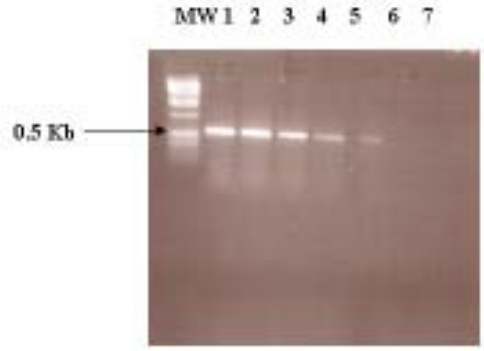

Fig. 1. Visualization of the 549-bp specific-PCR product on ethidium bromide-stained agarose gel from *M. paratuberculosis* DNA. Lane MW: molecular weight marker; lanes 1-5: 10 ng, 1 ng, 100 pg, 10 pg, 1.0 pg, respectively; lane 6: 10 ng of *M. tuberculosis*; lane 7: 10 ng *M. avium*.

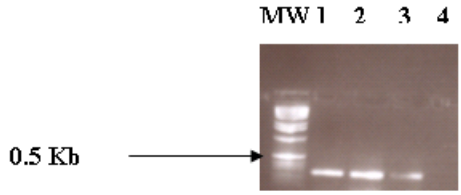

Fig. 2. Nested amplification showing specificity of the polymerase chain reaction for *M paratuberculosis*. Amplification product was not detected from less than 1 pg of *M.. paratubercullosis*. Lane MW: molecular weight marker; lane 1: 1 pg; lane 2: 100 fg; lane 3: 10 fg; lane 4: 1.0 fg *M. paratuberculosis*

The internal (nested) primers increased the sensitivity of the nPCR assay. The nPCR amplification produced a 260 bp PCR product from the first amplified product. The sensitivity of the assay was increased and the nPCR detected a lesser amount of DNA, which is equivalent to 10 femtograms (fg) of the total genomic DNA of the bacteria (Fig. 2).

Discussion

The diagnosis of Johne's disease is extremely difficult, especially in the primary stages. This is due to the long incubation period, the variable log phase associated with bacterial proliferation, and the multifocal distribution of slowly developing lesions (ABBAS et al., 1986). The diagnostic methods include serological and bacteriological cultures. Most of the serological reactions rapidly detect antibodies to *Mycobacterium paratuberculosis* but not active infections (MERKAL, 1984). The enzyme-linked immunosorbent assay (ELISA) was developed in the last few years to detect diseased animals. Conventional bacteriological isolations methods and identification are more accurate. However, they are cumbersome, rather expensive and time consuming, a procedure which normally takes 4 to 16 weeks (1-4 months) to grow.

Advance diagnostic procedures are currently used in many laboratories around the world. These procedures are based on molecular characterization of the specific bacterial isolates. These diagnostic tests use DNA probes, cloning, Restriction Fragment Length Polymorphisms (RFLPS) and PCR (MERKAL, 1984; SAIKI et al., 1989). The molecular biological methods increased the sensitivity, specificity and rapidity of detection of the organism. The PCR technique has found wide application. The results of this study showed that PCR could be used successfully to detect *M. avium* subspecies *paratuberculosis* from crude bacterial DNA extract. The PCR was able to amplify 549 bp-PCR products using primers derived from IS 1311. The sensitivity of the PCR assay was increased using internal primers (nested) amplification. A nested PCR product of a 260 bp-PCR product was amplified using internal primers. It is well documented that nested amplification increase the sensitivity of the first amplified product and is necessary to confirm the identity of the PCR product (ARADAIB et al., 1998a).

The specificity of this nPCR assay indicated that the specific primers used in this study did not amplify *M. tuberculosis*. DNA extracted from closely related acid-fast bacteria should also be applied to this nPCR for further investigation of the specificity of the PCR assay. The time required for amplification was approximately 3 hours. This finding suggests that tentative diagnosis of paratuberculosis infection could be based on visualization of the 549 bp PCR amplified product on an ethidium bromide-stained agarose gel, since it is a simple procedure and requires only 1 hour after amplification. The nested amplification of the 260 bp PCR product confirmed the specificity of the amplified product and increases the sensitivity of the PCR assay, particularly when the concentration of DNA in the sample is too small. The time required for nested PCR was also 3 hours. Thus, the time required from sample submission to interpretation of the final results was consistently 8 consecutive hours, which could be achieved within the same working day.

The described nPCR is an alternative to or supportive of the lengthy, cumbersome bacterial isolation procedures. The rapidity, sensitivity and specificity of the PCR assay would greatly facilitate detection of Johne's disease infection among susceptible ruminants in an enzootic location. Because the nested PCR amplification method is an extremely sensitive procedure, care must be taken to avoid cross-contamination between tubes during pipetting of reagents. Negative and positive controls should be included in each PCR reaction to estimate the lower limit of specificity and the higher limit of sensitivity.

Further studies are currently under way to determine the ability of the described nPCR assay to detect the organism in clinical samples from experimentally and naturally infected animals, and to evaluate its potential as a sensitive and specific diagnostic assay through comparison with current diagnostic test used for detection of paratuberculosis in susceptible ruminants.

Acknowledgements

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References

ABUBAKER, A. M., S. M. ELSANOUSI (1975): A survey of Johne's disease in Khartoum.Vet. Rec.18, 94-95

ARADAIB, I. E., M. A. ABDALLA, A. E. KARRAR (1998a): Application of biotechnology in diagnostic veterinary medicine. Proceeding of the 9th conference of Veterinary Medicine, Assiut University, Egypt pp. 362-386.

ARADAIB, I. E, C. E. SCHORE, J. S. CULLOR, B. I. OSBURN (1998b): A nested PCR for detection of North American isolates of bluetongue virus based NS1 genome sequence analysis of BTV-17. Vet. Microbiol. 59, 99-109.

ABBAS, B., S. E. O. IDRIS, A. BURHAN (1986): Isolation of *M. paratuberculosis* from goats in Sudan. The Sudan J. Vet. Sci. Anim. Husb. 25, 41-42.

BENDECTUS, G., A. A. DIJKHUIZEN, J. WAGEN (1987): Economic losses due to paratuberculosis in diary cattle. Vet. Rec. 121, 142-146.

FAWI, M. T., H. M. OBIED (1964): A note of Johne's disease among cattle in Sudan. Bull. Epizoot. Dis. Afr. 12, 437.

MERKAL, R. S. (1984): Paratuberculosis: Advances in cultural, serological and vaccination methods. JAVMA 184, 939-943.

MUNGASH, B. M. (1989): Diagnosis of Johne's disease in cattle. MSc Thesis, Department of Medicine, Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Khartoum, Sudan.

SAIKI, R. K., D. H. GELFAND, S. STOFFEL, S. J. SCHARF, R. HIGUCHI, G. T. HORN, K. B. MULLIS, A. H. ERLICH (1989): Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239, 487-491

SIGURDARDOTTIR, O. G., C. M. PRESS, F. SAXEGARD, O. VENSEN (1999): Bacterial isolation, immunological response, and histopathological lesions during the early subclinical of experimental infection of goats kid with *M. avium* subspecies *paratuberculosi*s. Vet. Pathol. 36, 542-50.

WHITTINGTON, R., I. MARCHALL, E. CHOY, D. COUSINS (1998): An insertion sequence common to *M. avium* and *M. avium* subspecies *paratuberculosis* can be used to distinguish between and with in these species. Mol. Cell Probes 12, 349-358.

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SAŽETAK

Mycobacterium avium subspecies *paratuberculosis* uzrokuje paratuberkulozu (Johneovu bolest) koja se očituje kroničnim tijekom u preživača. To je jedna od najraširenijih bakterijskih zaraza preživača diljem svijeta. Nedavno je objavljeno da je uzročnik povezan s crijevnom infekcijom u ljudi te je bolest postala važna za javno zdravstvo. U radu je rabljena ugniježđena lančana reakcija polimerazom (nPCR) za dokazivanje *Mycobacterium avium* subspecies *paratuberculosis*. Sekvencija 1311 je korištena kao ciljna DNK za unutarnju amplifikaciju pomoću lančane rekacije polimerazom. Najprije je određen par početnica (P1 i P2) od sekvencije s 1311 nukleotida za proizvodnju 549 pb PCR proizvoda. Zatim je upotrijebljen drugi par unutarnjih početnica (P3 i P4) za proizvodnju 260 pb PCR proizvoda. Ugniježđena reakcija je bila potrebna da poveća osjetljivost PCR-a i potvrdi identitet (specifičnost) prvotno umnoženog PCR proizvoda. Budući da je nPCR vrlo osjetljiva metoda može se koristiti za otkrivanje Johneove bolesti u ranom stadiju infekcije. Opisana nPCR metoda može se rabiti kao dodatna ili alternativna metoda u odnosu na uobičajene bakteriološke postupke koji se svakodnevno rabe za dijagnosticiranje bolesti u ljudi i/ili životinja.

Ključne riječi: *M. avium* subspecies *paratuberculosis*, Johneova bolest, nPCR