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STUDY OF ZOONOTIC TUBERCULOSIS CAUSED BY M. BOVIS BY POLYMERASE CHAIN REACTION

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SUMMARY

A polymerase chain reaction (PCR) assay was used to amplify a 248-bp sequence (IS1081) of M.bovis DNA using specific primers. Genomic DNA from M. bovis reference strains and M. bovis local strains were analyzed and yielded exclusively the 248-bp amplified fragment. The sensitivity of PCR detection was determined using different concentration of genomic M. bovis reference strains DNA. To evaluate the diagnostic ability of the assay for the detection of M. bovis in field samples, a total of 54 milk samples from tuberculin reactors cattle and in clinical samples, a total of 31 human sputum samples with suspected pulmonary tuberculosis were tested by PCR, direct smear and culture examination. In addition DNA amplification in non cultivated and cultivated sediments of milk and sputum samples was done and the positive results demonstrated by the presence of a single DNA fragment of 248-bp. Of 57 bacteriologically positive samples, 57 were positive by PCR and out 28 of culture-negative samples,1 gave a positive result by PCR. The PCR showed a sensitivity of (100%) and specificity (96.42%) when compared with bacteriological culture method. The specificity and sensitivity of PCR assay in detection of Mycobacterium bovis in field and clinical samples may provide a valuable tool for the rapid diagnosis and assists in the control of this zoonosis.

INTRODUCTION

Tuberculosis caused by M. bovis represents a major zoonosis and as a cause of economic losses, the infection is more in dairy cattle, not only because their useful economic life is longer, but because dairy cattle are in closer contact with one another during milking (Acha & Szyfres, 1989). Pulmonary as well as extra pulmonary

forms of human tuberculosis of animal origin continue to be a problem in areas where the prevalence of infection in cattle is high, because not all milk consumed is boiled, many products are prepared from unpasteurized milk, and in addition cases of infection are contracted via aerosols (Zanini et al. 1998). Laboratory diagnosis of mycobacteria by Ziehl-Neelsen stain to identify acid fast bacilli (AFB) is lacks sensitivity and specificity, this method requires at least 10^4 to 10^5 bacilli per ml in the sample for a positive result, many specimens containing lower quantities of bacilli are recorded as negative. Thus patients with fewer than 10^4 bacilli per ml are not diagnosed and remain contagious (Densie et al. 1993). On the other hand it takes 4 to 8 weeks to culture pathogenic mycobacteria because of their slowly growing nature (Daniel, 1990). New possibilities are disclosed by PCR assay based on nucleic acid technology, which are able to identify in a short time a few thousand units to a few units of mycobacterial cells (Bollo et al. 1998).

Many primers which amplify specially the DNA of mycobacteria have been designed and successfully used for identification of this m. o. from culture (Eisenach et al. 1990) and also from clinical samples (Brisson-Noel et al. 1991). DNA fingerprinting analysis requires the use of greater safety precautions because standardized analytic procedures are not completely safe for mycobacteriology laboratory staff. A case of pulmonary tuberculosis was reported to be acquired in laboratory technician who had no apparent risk factor. So it must be ensured that cultures are really inactivated by boiling before DNA processing (Bermer-Melchior & Drugeon, 1999).

The heating time seems to be more important than the heating temperature in preserving the integrity of the mycobacterium DNA, (Zwadzk et al. 1994) who showed that genomic DNA is sheared into small pieces if it is heated for 30 minutes. This study was planned to apply the PCR assay on culture of M. bovis after boiling at 100°C for 10 minutes then used directly for DNA amplification without any further processing and to determine its utility in the diagnosis of zoonotic tuberculosis in field and clinical samples.

MATERIALS AND METHODS

Bacterial Strains: M. bovis reference strains originated from Central Veterinary Laboratory, Weybridge, UK and M. bovis local field isolates from cows milk were supplied from Mycobacteria Dept. Vet. Sera and Vaccine Institute, Abbassia. Bacteriological examination of strains by direct smear method, culture on Lowenstein Jensen medium and biochemical identification according to Koneman et al. (1992).

Polymerase Chain Reaction Assay: -

Oligonucleotide primers: The primers used BW8-BW9 belonging to an insertion sequences (IS 1081) in strains of M.bovis for amplification

248-bp. The DNA sequence are (BW8) 5`-ACA, GGC, GAG, CCC, GGA, TCT, GCT G-3` and (BW9) 5`-GTT, CAG, CTC, GCT, TGC, GGC, GCT G-3` according to Wards et al. (1995). The specific primers were supplied from Difco Detroit, Michigan, USA. A 100-bp DNA was used as molecular size marker.

Preparation of template DNA: -

Carried out after (Kocagoz et al., 1993) for preparation of template DNA, a suspension of M. bovis having 10^5 microorganisms per ml was prepared in TE buffer (10 mM Tris (pH 8), 1 ml M EDTA), by adjusting its turbidity with McFarland standards. A total of 10 ul of this suspension was put into Eppendorf tube, placed on boiling water bath for 10 minutes to breakdown the bacterial cell wall and release DNA and then used directly for amplification without any further processing.

PCR procedure for Amplification of DNA:

Outlined after (Zanini et al. 1998). Amplification reaction mixtures were prepared in a volume of a 25ul containing (50 mM KCl, 10 mM Tris pH 8.3, 20 mM MgCL2, 0.2 mM (each) of dATP, dCTP, dGTP & dTTP (Difco) and 0.5 uM each primer BW8-BW9, 0.5U of uracil DNA glycoslase, 2.5U Taq polymerase (Difco) and 1-20 ng of M. bovis DNA. The DNA was denatured for 10 min at 95°C. The mixture was then subjected to 42 cycles of amplification by denaturation at 95°C/ 30s, annealing of primers at 68°C / 60s and primer extension at 72°C for 30s. The final extension was held at 72°C for 30 min. A negative control sample containing saline instead of template DNA was included with each PCR to exclude the possibility of reagent contamination.

Identification of PCR product by agarose gel electrophoresis:

The presence of the 248-bp amplified product was analyzed by electrophoresis of 10 ul of the amplified mixture on a 2% agarose gel. The DNA was stained by ethidium bromide and photographed on an ultraviolet transilluminator and compared with molecular size marker as described by (Kocagoz et al. 1993).

Determination of sensitivity of PCR:

The sensitivity of PCR assay was determined by using 10-fold dilutions of the purified genomic DNA from M. bovis reference strains and local isolates. DNA was prepared by just boiling the organisms. Tubes containing 10^8-10^1 CFU / ml were placed on boiling water bath for 10 minutes, and a 10 ul from each tube was used for amplification as described above.

PCR testing and bacteriological examination of field and clinical samples:

Sampling: Milk samples were aseptically collected from 54 cattle positive reactors to the intradermal tuberculin test. Tuberculin test was carried out after El-Taweel (1992) by using

purified protein derivative (PPD) of M. bovis provided from (Vet. Sera and Vaccine Institute, Abbassia). As well as 31 sputum samples were collected from patients with symptoms of pulmonary diseases and admitted to the Chest Hospital in Benha and El-Abbassia districts. Data were collected after clinical diagnosis of pulmonary tuberculosis by patient histories, clinical and radiological findings and response to antituberculosis drug therapy. Milk and sputum samples were transported to the laboratory without delay and under aseptic conditions were decontaminated with N-acetyl-L cysteine-NaOH (NALC-NaOH) method according to (Kubica et al. 1963) and centrifuged at 1500 r.p.m. Sediments to be tested by PCR were stored at –20°C, while those used for bacteriological investigation were kept at 4°C until being tested within 24 hours (Abe et al. 1993). Direct smears prepared from the sediment and stained with Ziehl-Neelsen and examined for acid fast bacilli (Luna, 1968).

The sediments were suspended in 1 ml of phosphate buffer (pH 7.4), 0.3 ml of suspension was inoculated into Lowenstein Jensen medium (Difco) and incubated for 10 weeks. Identification of the organism based on growth rate, colony morphology, pigmentation and biochemical test (niacine, nitrate reduction, 68°C catalase, tween hydrolysis and urease) according to (Koneman et al. 1992).

Specimen processing and DNA amplification: Carried out after (Kocagoz et al. 1993). The sediments of milk and sputum samples were washed three times by centrifugation in an Eppendorf microcentrifuge at (12.000 r.p.m.) and resuspension in TE buffer to the protein & salts produced by decontamination with NALC-NaOH. The final sediment was resuspended in 50 ul of TE buffer, and the tube was placed in boiling water bath for 10 minutes. A 10 ul was used for PCR. In addition to preparation of template DNA in cultivated sediments of milk & sputum samples was done as described before. The DNA amplification in non-cultivated and cultivated sediments of milk and sputum samples was performed by the above described procedure. The sensitivity and specificity of the PCR assay as a diagnostic test were calculated as described by (Monke et al. 1992). To define sensitivity and specificity, results of the PCR were compared with those of bacteriological cultural method. 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 AND DISCUSSION

PCR amplification of DNA from M. bovis reference strains & local isolates:

Fig. (1) shows the results of the PCR amplification of M. bovis DNA from reference strains and local isolates using the BW8 and BW9 primers. As predicted, one band of the expected size (248-bp) was amplified from the DNAs of M. bovis reference strain, while local isolates yielded identical amplified DNA bands of the same molecular size marker on agarose gel but not seen in negative control sample. This result substantiates that obtained by (Zanini et al. 1998). Preparation of mycobacterial DNA by just boiling at 100°C / 10 minutes rather than enzymatic lysis by using proteinase K and incubated at 37°C / 3 hrs followed by phenol-chloroform extraction was better for amplification with PCR . This might be due to the loss of some of the DNA during purification step in the latter method (Kocagoz et al. 1993).

Sensitivity of PCR in detection of Mycobacterium bovis:

The purpose of the pre-evaluation study was to ascertain the reliability of PCR for the identification of M. bovis. To determine the sensitivity of mycobacteria DNA detection by PCR amplification, a 10-fold dilution series from 10^8-10^1 CFU / ml of M. bovis reference strain purified genomic DNA was subjected to PCR. Fig. (2), shows the results of agarose gel electrophoresis of PCR product following amplification of different M. bovis genomic DNA dilution tested as detected by visualization on agarose gel. These obtained results were in accordance with those detected by (Kocagoz et al. 1993). Moreover PCR assay can detect fewer than 10 m.o. / ml (Abe et al. 1993).

PCR testing and bacteriological examination of field and clinical samples:

To assess the utility of the PCR assay in the detection of M. bovis in milk samples from positive tuberculin cows and in human sputum of clinically suspected pulmonary tuberculosis were tested by PCR, direct smear examination and culture method. As shown in fig. (3) M. bovis DNA was detected by PCR amplification in non-cultivated and cultivated sediments of milk and sputum samples, the positive result demonstrated by the presence of a single DNA fragment of 248-bp. A result which is similar to that demonstrated by (Kocagoz et al. 1993). PCR technique amplifies the nucleic acid from the cultivated and non-cultivated infectious agents. Moreover it amplifies DNA from a variety of sources can be applied including, paraffin embedded tissue, dry frozen or wet preserved wet tissue over many thousand years old which can be help in solving many problems of public health importance (Bollo et al. 1998).

The results of PCR and culture procedure were compared as shown in table (1). Of 57 culture

Table (1): Sensitivity & specificity of PCR in comparison to the culture method.

Method	No.	PCR			
		+ve	-ve	Sensitivity %	Specificity %
Culture+ve	57	57	--	100	--
Culture -ve	28	1	27	--	96.42

positive samples, 57 were positive by PCR and out of 28 culture negative samples, 1 gave a positive result by PCR. The sensitivity of the PCR assay was 100% and its specificity was 96.42% as compared with culture technique. A finding which is almost similar to that obtained by (Abe et al. 1993).

Beside its reliability, high sensitivity and specificity, several advantages over the microbiological diagnostic method for M. bovis. A major advantage is the speed with which the assay can be performed, where as results could be obtained within less than 24 hrs. Conventional methods require at least, several weeks. Moreover, it eliminates the hazards of handling mycobacteria in the laboratory.

From zoonotic point of view, the recent development of nucleic acid technology provides rapid, accurate & sensitive tool, which can significantly contributes both to a better understanding of the epidemiology of tuberculosis and to programmes for control and eradication of this zoonosis.

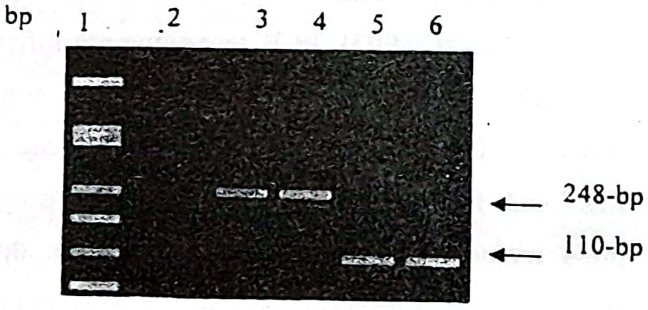

Fig. (1): PCR amplification products from M. bovis reference strains and local isolates tested with BW8-BW9 primers. A 10ul sample of each PCR product was resolved by electrophoresis in agarose, visualized by ethidium bromide. Lane 1 : a 100 – bp molecular size marker , Lane 2 : a negative control sample, Lane 3,4 : M. bovis reference strains, Lane 5, 6: M. bovis local isolates from cow milk.

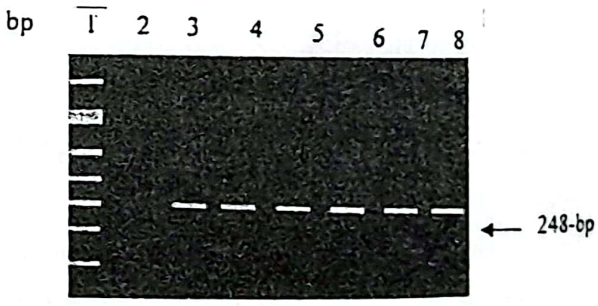

Fig. (2): Sensitivity of PCR amplification for the detection of DNA from M. bovis reference strain. Ten fold serial dilutions from 10^{8}-10^{1} CFU / ml of purified genomic DNA were analyzed by PCR. A 10 ul sample of each PCR product was run on a 2% agarose gel, visualized by ethidium bromide. Lane 1: a 100 - bp molecular size marker, Lane2: a negative control sample, Lane 3 – 8: serial M. bovis DNA concentrations indicated 248-bp amplified fragment.

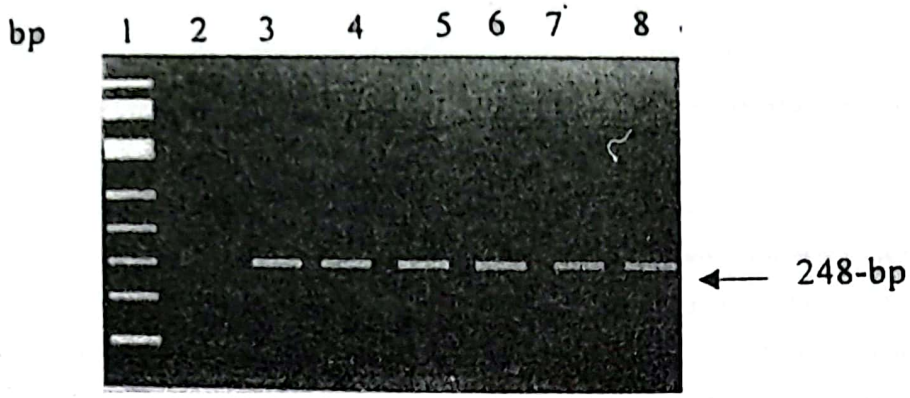

Fig. (3) Amplification products (248-bp) DNA by PCR from the tested non-cultivated and cultivated sediments of cow milk and human sputum samples. Lane 1: a 100-bp DNA molecular size marker, Lane 2: a negative control sample, Lane 3, 4: cow milk samples, Lane 5, 6: human sputum samples, Lane 7, 8: M. bovis from cultural isolated from the field and clinical samples respectively

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