Identification and Differentiation of Mycobacterium Avium Subspecies Paratuberculosis Isolates Using and pAM-3 as a DNA Probe

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Abstract

Background: *Mycobacteriu avium subsp.paratuberculosis* (MAP) is the causative agent of Johne's disease. It is a very slow growing bacterium on synthetic medium. The use of conventional methods for diagnosis is time consuming and not accurate.

Objectives: The use of molecular biological techniques for fast and accurate diagnosis of *Mycobacterium avium subsp.paratuberculosis* isolates.

Materials and methods: DNA was extracted and prepared from four *Mycobacterium avium subsp.paratuberculosis* and four *Mycobacterium tuberculosis bovis* isolates. The extracted DNA was subjected to PCR by using specific primers and the isolates were distinguished and differentiated by hybridization technique using pAM-3 specific probe developed in New Zealand.

Results: The PCR products gave the requested DNA fragment of 163 base pairs. The probe reacted specifically with targeted DNA fragment of paratubreculosis bacterial isolates but not with tuberculosis bovis isolates.

Conclusion: The use of PCR and specific DNA probe for the diagnosis of MAP is a fast and accurate method for diagnosis of MAP.

Keywords: Mycobacterium, Paratuberculosis, Johne's disease.

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Introduction

John's disease that caused by *Mycobacterium avium subspecies paratuberculosis* (MAP) was regarded as one of the important diseases that affect animal industry in Malaysia [1] and worldwide [2]. It is well known that contaminated materials, semen, saliva, uterine fluid, food, aerosol are the major transmission modes of infection to ruminants [3, 4, 5]. Cattle, deer, sheep and goats are not the only infected animals [6, 7] but also wildlife animals were susceptible (8, 9). New borne animals were more susceptible to the infection than adult and this was attributed to the stage of development of innate and acquired immunity [10, 11].

The diagnosis of MAP depends mainly on isolation of bacteria followed by several

biochemical tests, which are normally taking a longtime,

With advance of molecular biology, several tests have been developed to accurate and reduce the time required for the diagnosis of those slowly growing pathogenic bacteria [12- 14]. One of them is the use of amplification system in parallel with biotechnology and molecular genetics which has greatly assist in making fast diagnosis [15-17]. A cloned of DNA probe (pAM-3) was developed in the gene library of *E.coli* expression vector phage λgt11, for identification and differentiation of MAP in New Zealand [15].

In this study, we used the pAM-3 probe developed in Massy University, New Zealand for identification of some isolates of MAP.

Materials and Methods

Enzymes and Chemicals

PCR reagent kit (GeneAMP^R) with Taq enzyme is obtained from Perkin Elmer Cetus^R. Proteinase k is obtained from Bethesda Research Lab., USA. Primer 1 (TDB-3), primer2 (TDB-4), pAM-3 probe and DNA of MAP as a positive control were kindly provided by Dr. Alan Murray (New Zealand). Non-radioactive labeling was done by using Sulfoprobe-3 kit (Sigma Chemical Company, USA). Nylon membrane (Hybond TM-N) was obtained from Amersham International, Amersham, U.K.

Bacterial Strain

Four MAP isolates grown in Lowenstein medium (referred as IPH1 to IPH4) and four Mycobacterium tuberculosis bovis (MTB) isolates grown on Stonebrink's medium referred as IPH5 to IPH8 were kindly provided by Animal Health Institute, Ipoh, Perak, Malaysia.

DNA Extraction

Five loopfull of bacterial growth on solid medium were collected and suspended in 500 µl of 50 mM Tris-HCl buffer pH 7.8 in 1.5 ml eppendorf tubes. The bacterial cells were pelleted by 3000 rpm for 20 minutes, washed three times with same buffer, freezed and thawed several times and followed by DNA extraction and ethanol precipitation according to the method described by Perbal (18).

DNA Preparation from bacterial cells

Two loopfull of bacterial growth of each sample collected, suspended, pelleted and washed as above and processed for polymerase chain reaction according to the method described by Kawasaki (19).

DNA Amplification by Polymerase Chain Reaction (PCR).

PCR was carried out on 5µl of DNA extracted and 10µl of DNA prepared from each bacterial cell samples with 1 µl of primer 1 and 2 in PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM Potassium chloride, 1.5 mM Magnesium Chloride, 0.001 percent w/v gelatin, 200 µM of each dATP, dGTP, dCTP and dTTP). This was followed by addition of 2.5 units of Taq polymerase. The total volume was adjusted to 100 µl of PCR mixture (20).

The reaction also contained three controls: 100 µl of 1x PCR buffer only, PCR reaction mixture without target DNA and PCR reaction mixture with DNA of MAP (New Zealand strain) as positive control.

Ten µl from each sample and control were collected as pre PCR control, and the remnant of each reaction mixture overlay with one µl of mineral oil.

Extracted DNA and prepared samples with controls were amplified for 35 cycles using DNA thermal cycler 480 of Perkin Elmer^R and each cycle consisting of 94ºC for one minute (denaturation), 55ºC for one minute (annealing) and 72ºC for one minute (extension).

The pre PCR aliquots and PCR products were electrophoresed in 2% agarose prepared in TAE running buffer containing 0.05 µg/ml ethidium bromide and run for 1 hour at 100 volts. Furthermore, Ø Bacteriophage 174

DNA fragments were used as base pairs size markers. The size range of marker from 72, 118, 194, 234, 271, 310, 603, 872, 1078, and 1353 base pairs. The results were visualized through UV source and photographed by Polaroid film 667.

Southern Blotting and Hybridization of PCR product.

Blotting, prehybridization and hybridization were carried out on nylon membrane according to the methods described by the manufacturer (Amersham, International, and UK).

Labeling of pAM-3 probe with non-radioactive sulphoprobe-3, visualization of the sulphoprobe in the hybrids and the visualization system control were carried out according to instruction manual of sulphoprobe kit^{R}.

Results

The PCR products of DNA prepared from the MAP which were electrophoresed in 2% agarose gel showed a clear band of 163 base pairs was shared between the positive control DNA of MAP of New Zealand strains with that of four MAP of Malaysian isolates (Plate-1). Furthermore, such band was not seen in all MTB isolates (Plate-2).

Extracted DNA also gave the same results except for the IPH3 isolate which showed a very faint bands (Plate 3 and 4).

The two primers (TDB-3 and TDB-4) used in PCR lead to the formation of two bands in the DNA of positive control MAP as well as many bands in both MAP and MTB of Malaysian isolates.

Hybridization and immunological visualization of PCR products of both MAP and MTB isolates with positive controls revealed that pAM-3 probe, clearly hybridized to the PCR product of MAP isolates and positive control only (Plate-5 and 6).

Plate (1): PCR products of DNA prepared from Malaysian isolates of MAP isolates (IPH) run in 2% agarose gel. Lane 1: Bacteriophage 174 as a DNA molecular weight marker; Lane 2 Positive control of 163 bp; Lane 3: Negative control 1; Lanes 4, 5, 6 and 7: IPH1, IPH2, IPH3 and IPH4 of MAP respectively; Lane 8 Negative control 2.

Plate (2): PCR products of DNA prepared from Malaysian MTB bovis isolate (IPH) run in 2% agarose gel. Lane 1: Bacteriophage 174 DNA marker; Lane 2: Negative control; Lanes 3, 4, 5 and 6: IPH5, IPH6, IPH7 and IPH8 respectively; Lane 7 Positive control of 163 bp.

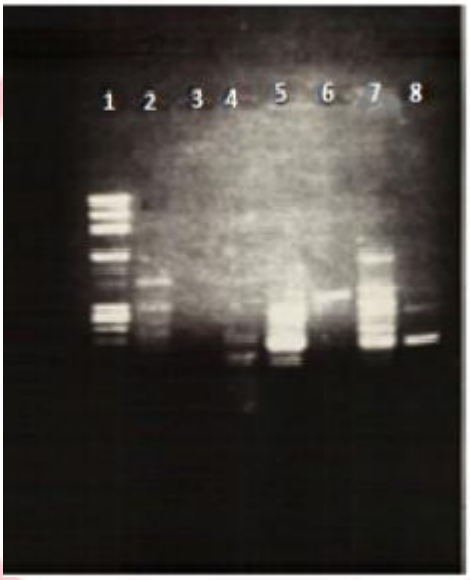

Plate (3): PCR products of DNA extracted from Malaysian MAP isolates (IPH) run in 2% agarose gel. Lane 1: Bacteriophage 174 DNA marker; Lanes 2 and 3: Negative control: lanes 4, 5, 6 and 7; IPH1, IPH2, IPH3 and IPH4 respectivilely; Lane 8 Positive control of 163 bp.

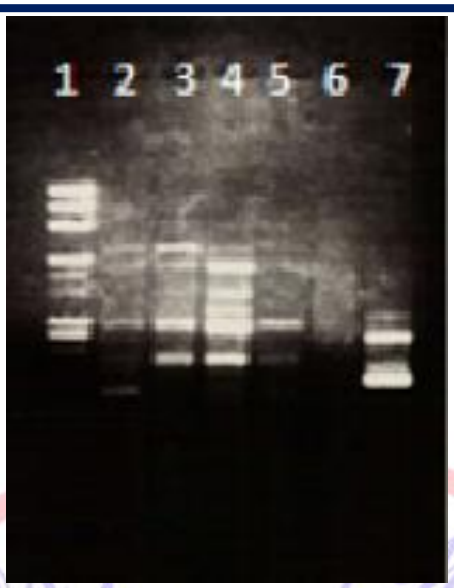

Plate (4): PCR products of DNA extracted from Malaysian MTB bovis isolates (IPH) run in 2% agarose gel. Lane 1: Bacteriophage 174 DNA marker; Lanes 2, 3, 4 and 5 are IPH5, IPH6, IPH7 and IPH8 respectively; Lane 6 Negative control; Lane 7 positive control.

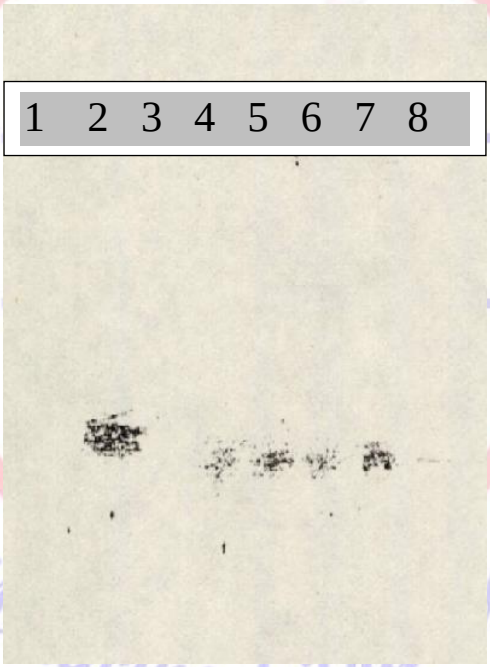

Plate (5): Hybridization and Immunological Visualization of PCR products of Malaysian MAP DNA with the non-radioactive sulphoprobe. Lane 1 Ø Bacteriophage 174 DNA marker; Lane 2 Positive control; Lane 3 Negative control; Lanes 4, 5, 6 and 7; IPH1, IPH2, IPH3 and IPH4 respectively; Lane 8 Negative control 1.

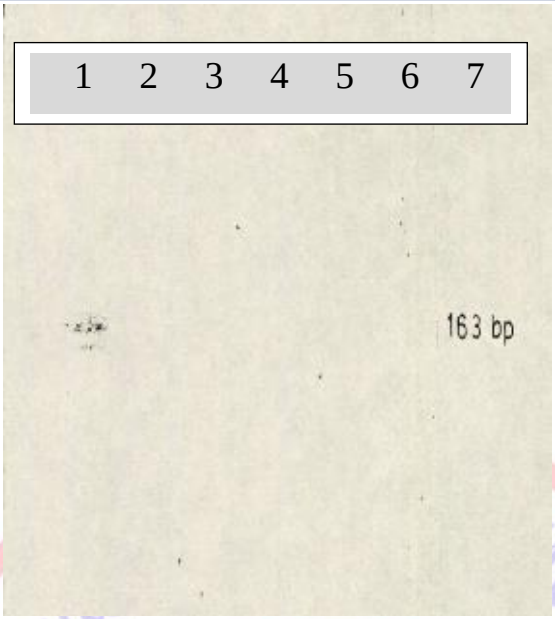

Plate (6): Hybridization and Immunological visualization of PCR products of Malaysian MTP bovis DNA with the non-radioactive sulphoprobe. Lane 1 Positive control; Lanes 2, 3, 4 and 5: IPH5, IPH6, IPH7 and IPH8 respectively; Lane 6 Negative control; Lane 7 Ø Bacteriophage 174 DNA marker.

Discussion

The use of TDB-3 and TDB-4 primers in PCR analysis lead to formation of specifically shared band between the positive control and MAP isolates and the results can easily and simply distinguish these isolates from MTB bovis. Polymerase Chain Reaction has been used by many researcher to focus on certain gene fragment of MAP to be used for diagnosis of differentiation between MAP strain s or other Mycobacterium [6, 14,16, 21, 22 and 23]. Presence of many PCR DNA bands in MAP isolates in comparison to positive DNA control may be attributed to improper optomization of PCR [24] or strain variation [25, 26,27,28,29 and 30]. Generally, MAP isolates were classified as cattle, sheep and bison types [31] or into cattle (C) and sheep(S) depending on the virulence factor heparin-binding hemagglutinin adhesin (HBHA) [32]. classification of MAP into type (I) for cattle and type (II) for sheep also has been mentioned by many authors [13,33]. This study successfully used hybridisation technique with a specific probe pAM-3 to identify and to differentiate MAP isolates from MTB bovis isolates. The use of specific probes to differentiate between MAP isolates and other Mycobacterium have been used by many researchers [17, 21 and 34]. This hybridization of labelled probe technique had also been reported to be able to identify and differentiate between bacterial isolates that are pathogenic and /or non-pathogenic [15, 35 and 36].

In a final conclusion using of specific DNA probe against MAP isolates is a useful for rapid diagnosis of slowley growing pathogenic bacteria. It reduced the time and appeared to be more accurate dignostic procedure. We suggested further study on the use of restriction field length polymorphism (RFLP) for both MAP, MTB and other mycobacterium species to point out the similarity and dissimilarity betrween the isolates especially those of zoonotic activity to prepare rapid and highly accurate diagnostic tools.

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