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Identification of the *Mycobacterium spp*. Isolated from Cows Milk Samples by Using PCR Technique

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Abstract

The aim of this study was to identify the Mycobacterium isolates to species level by application of molecular method (polymerase chain reaction-PCR) with the specific primers that can differentiate the DNA of *Mycobacterium tuberculosis* and *Mycobacterium bovis*. Mycobacterium isolates used in this research were obtained from mastitic and apparently normal milk samples from many areas of Baghdad province in previous study. The isolates were previously identified as Mycobacterium based on direct identification of acid fast bacilli, cultural and biochemical properties. For differentiation of *M.bovis* from other species of Mycobacterium we used three sets of primers, these primers included a common forward primer 5'-TTCCGAATCCCTTGTGA-3', coded CSB1, reverse primer 5'- GGAGAGCGCCGTTGTA-3', coded CSB2 (*M.bovis*) and reverse primer 5'- AGTCGCGTGGCTTCTCTTTTA-3', coded CSB3 (*M.tuberculosis*). The m-PCR result showed that thirteen isolates out of fifteen was *M.bovis* as the product was 168bp and 2 isolates were *M. tuberculosis* complex as the product was 337bp .This research indicates that the m-PCR method using specific primers (CSB1, CSB2, and CSB3) can be applied for identification of *M. tuberculosis* and *M. bovis* and the importance of the implementation of measures for the prevention of tuberculosis in both humans and animals.

Keywords

Mycobacterium tuberculosis, *M. bovis*, Milk, Multiplex Polymerase Chain Reaction (m-PCR), Cow

1. Introduction

There is increasing contact between humans and animals worldwide due to increasing population density and growth especially in poor developing countries where livestock offer important socioeconomic pathway out of poverty (1). Animal and human tuberculosis (TB), emerging or reemerging and caused by pathogenic bacteria of the *M. tuberculosis* complex, *M. tuberculosis* and *M. bovis* are the major causes of tuberculosis, which are highly pathogenic that may infect many animal species and thus are likely to be the source of tuberculosis infection in humans. (2,3,4,5,6,7).

Human TB is mainly caused by *M. tuberculosis* but in regions where bovine TB is prevalent in animals, human TB cases due to *M. bovis* may occur (8,9) resulting from ingesting contaminated unpasteurized milk and also by inhaling cough spray from infected livestock (5). *M. bovis* remains an important veterinary disease, with almost 200000 infected cows among a total cattle population of ~170 million in America and Caribbean (10). This organism also poses human public health problems as well because it is suspected that *M. bovis* infection is responsible for approximately 4000 of the approximately 80000 cases of human tuberculosis reported each year in Brazil (11, 12).

Mycobacterium bovis is a member of the *M. tuberculosis* complex, which mainly affects cattle and secondarily other mammals. In humans, *M. bovis* produces infection that is clinically indistinguishable from tuberculosis (TB), Moreover, disease caused by *M.bovis* in human Immunodeficiency virus (HIV) positive individuals is also an increasing concern (13, 14).

There is a direct correlation between *M. bovis* in cattle and disease in the human population (5). In industrialized countries, animal TB control and elimination programs, together with milk pasteurization, have drastically reduced the incidence of disease caused by *M.bovis* in both cattle and human but in developing countries, however, TB is widely

distributed, control measures are not applied or are applied sporadically, and pasteurization is rarely practiced (15).

For instance, *M. bovis* infection is responsible for about 2% and 8% of new cases of human pulmonary and extra pulmonary TB, respectively, in Latin-America(5). In Asia, 94% and less than 99% of total cattle and buffalo populations, respectively, are found in countries where bovine TB is either partly controlled or not controlled all (12). Thus, 94% of the Asian human population lives and is at a risk, in countries where cattle and buffaloes undergo no or only limited control for bovine TB (5).

The disease remains a major public health issue ,in 2004 mortality and morbidity statistics included 14.6 million chronic active cases, 8.9 million new cases, and 1.6 million deaths, mostly in developing countries with an expected 1% increase annually (15). Iraq one of the high TB burden countries in the Eastern Mediterranean region, with the highest tuberculosis burden, the estimated incidence of all TB forms accounted for 56/100000 population in 2008 (16).

Diagnosis need a lot of time, hence, molecular method was used to identify this microorganism. PCR technique was used as one of the effective molecular method for identification the microorganism to the species level (17).

2. Materials and Methods

2.1. Bacterial Isolates

Fifteen isolates of *Mycobacterium spp.* were previously isolated from cow's milk, diagnosed by conventional method, based on culturing on stone-brinks media and biochemical properties in niacin test, nitrate reduction, catalase test, and growth on Lowenstien-Jenseen (LJ) slant with sodium pyruvate (18). These cultures were harvested with phosphate buffer saline, pH 7.2 (PBS) and centrifuged (gallenkamp) at 3000 rpm for 15 minutes. The supernatant was discarded; the sediment was taken for DNA extraction.

2.2. DNA Extraction Procedure

For the extraction of PCR amplifiable DNA, a loopfull of mycobacterial sediment was suspended in microfuge tube containing 400 ul of 1X TE (Promega) (10uM Tris – HCl, 1uM EDTA, PH 8.0) the suspension was then subjected to boiling for 10 min at 95^{o}C, 200 ul of the solution used for DNA extraction by using DNA extraction kit (Sacace) and then the 10 ul of extracted DNA used for PCR (19).

2.3. Primers and PCR Conditions

Oligonucleotide sequences of the primers used in the study were:

The common forward primer, CSB1 (5′-TTCCGAATCCCTTGTGA-3′), and two reverse primers, including *M.bovis* specific, CSB2 (5′-GGAGAGCGCCGTTGTA-3′) and *M.tuberculosis* – specific, CSB3 (5′-AGTCGCCGTGGCTTCTCTTTTA-3′) (Alpha. Canada).

The m-PCR reaction was performed in a total volume of 50ul consisting of the following:

5ul of template DNA, 2ul from each primer (CSB1, CSB2, CSB3), 25ul master mix (promega), and 14ul D.W. (19)

2.4. The Cycling Parameters

The cycling conditions were initial denaturation at 94°C for 5 minutes, followed by 30 cycles of a denaturation step at 94°C for 1 minute, primer annealing at 52.3°C for 1.30 minutes, extension at 72°C for 1 minutes and final extension at 72°C for 5 minutes.

2.5. Gel Electrophoresis

Two percent 2% agarose was made by adding 2g of agarose (Difco) to 100ml of 50X TAE buffer and then it was solubilized by heating in boiling water bath, the agarose was left to cool at 50-60^{o}C before adding the ethidium bromide (Promega) and pouring the gel, 10ul of ethidium bromide (10 mg/ml- stock solution) was added to the agarose. Then the gel was poured in the tray and fixed the comb at the right position and left until solidifying. Then the comb removed carefully, 3- 5 µL of PCR product with loading buffer was added into each comb well.Then the tray transferred into electrophoresis machine which contained the same buffer that used in preparation of agarose gel Then an electric current was performed at 80 volt and 80 AM for 1hr. Finally PCR products (bands) were visualized using a UV transilluminator and photographed by using digital camera.

Ethics: This study was approved by the Ethical and Research Committee of the College of Veterinary Medicine – University of Baghdad.

3. Results

The m-PCR assay was applied to DNA from 15Mycobacterium strains isolated from normal and mastitic cow's milk samples, 13 isolates were positive for the 168-pb fragment specific for *M. bovis* and 2 isolates were positive for the 337-pb fragment specific for *M. tuberculosis* (Fig.1).

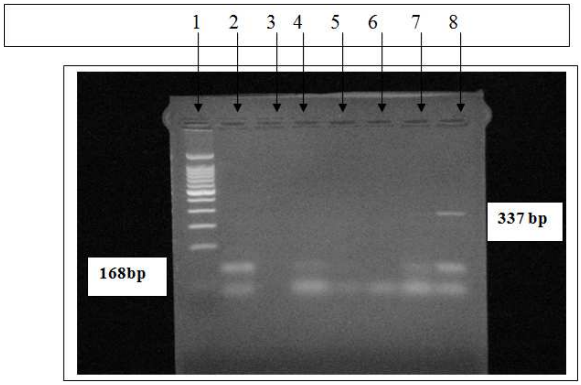

Figure 1. *1-marker 2-positive M.bovis 3-negative 4-positive 5-negative 6-negative 7- positive M.bovis 8- positive M.tuberculosis.*

4. Discussion

Mycobacterium tuberculosis and *M. bovis* are the major

causes of tuberculosis. These may infect many animal species, and are likely to be the main source of infection in humans (19). Conventionally, microorganisms that cause tuberculosis were diagnosed based on acid fast stain, culturing, and biochemical methods. The isolation and identification of slowly growing pathogens like mycobacterium entails a period of several weeks. Bacteriological isolation is a time consuming procedure and handling the microorganism is hazardous (20, 21). Despite progress in biochemical, biological, and immunological techniques, the reliable identification of my cobacteria remains a problem (22, 23).

Several alternative methods have been attempted for the rapid and specific diagnosis of tuberculosis, but molecular approaches, especially polymerase chain reaction (PCR) assays, are the most promising for direct detection of the etiological agent (24, 25).The utility of the m-PCR assay based on the CBS1, CBS2 and CBS3 primers has been earlier shown to differentiate *M.bovis* from *M.tuberculosis* with high sensitivity and specificity for the *Mycobacterium* (19).

We used the same 3 sets of primers to differentiate between *M.bovis* and *M.tuberculosis* and the results exhibits that 13 isolates of 15 isolates were positive for the 168-pb fragment specific for *M.bovis* and 2 isolates were positive for the 337-pb fragment specific for *M.tuberculosis*. Similarly many researchers reported the isolation of *M.bovis* and *M.tuberculosis* from cow's milk samples (2, 18). Identification of *M. bovis* in bovine samples has become necessary, as infected animals are potentially capable of infecting humans (zoonotic tuberculosis) (5), *M. bovis* is the etiological agent of bovine tuberculosis which causes economic and public health problems in many countries (5,6).

The detection of both pathogens in the cow's milk point out a twin danger of infected cattle being a source of mycobacterial pathogens capable of infecting animals and humans. Contact between farm animals and humans would naturally facilitate a bidirectional infection with these pathogens (17).

Humans are the initial source of *M. tuberculosis* infection for animals, and there are potentials for this infection being carried back to humans. The transmission of *M. tuberculosis* from infected humans to animals and back has been reported for animals in contact with humans (reverse zoonosis) (26, 27). Recent articles have reported isolation of *M. tuberculosis* in cattle with prevalences of 4.7%–30.8% in African and Asian countries (28). Grange and Yates (29) reported that farm workers urinating in cowsheds might constitute a source of infection for animals. Animals infected with *M. tuberculosis* represent a potential risk of transmission of virulent tubercle bacilli back to humans (27). Therefore, there is a compelling need to diagnosis tuberculosis among animal handlers and farmers, as humans are the major reservoir of *M. tuberculosis* (17).

The identification of *M.bovis* and *M.tuberculosis* in cow's milk samples as determined by PCR assay would suggest redefining the existing control and prevention policies for tuberculosis. Hence, the present study along with other reports in Iraq (2, 18) require the coordination effort of medical and veterinarian health professionals to define and implement unified control measures for prevention of both human and animal's tuberculosis.

Many researchers have compared classical diagnostic methods with PCR for diagnosis of different microorganisms, and reported that PCR was a more sensitive method (24, 25, 30).

In conclusion, the m-PCR assay used in this study was highly effective in differentiation of *M.bovis* from of *M.tuberculosis* and tuberculosis can be transmitted to people as a consequence of drinking contaminated milk. Therefore, this assay would be of immense utility in defining research priorities and public health strategies for control and prevention of tuberculosis in humans as well as in farm animals, since the *M. tuberculosis* and *M. bovis*, is capable of infection and of causing both human and bovine tuberculosis.

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