Nested polymerase chain reaction as a molecular tool for detection of Mycobacterium tuberculosis complex recovered from milk samples

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INTRODUCTION

Mycobacterium bovis, the causative agent of tuberculosis in cattle, is also a pathogen for a large number of other animals, and its transmission to humans constitutes a public health problem (World Health Organization, 1999; Ayele et al., 2004; Cedeño et al., 2005). The number of M. bovis positive cattle has been shown to be on the rise in the past 10 years. Epidemiologically, the main cause of such increase are the. Importation of infected animals, incomplete removal of infected cases and movement of Mycobacterium exposed animals between herds (Mishra et al., 2005). Although pasteurization has drastically reduced the transmission of M. bovis from cattle to human, the increasing incidence in cattle make exposure of human population to M. bovis more likely (Santos et al., 2010). Bovine tuberculosis is generally transmitted to human from animals in three main ways, inhalation of infected droplet nuclei containing M. bovis; ingestion of contaminated materials usually milk and directly among workers who are in direct contact with the diseased animals. The diagnosis of bovine tuberculosis in farm
MATERIALS AND METHODS

Animals

A total of 3255 cattle and 2650 buffaloes during the period of 2008 to 2010 from different Egyptian Governorates were tested with mammalian tuberculin. Tuberculin-positive reactors (105 cattle and 85 buffaloes) were slaughtered and from such animals, lymph nodes, milk samples and serum samples were collected.

Milk and serum sample collection

A total of 520 milk samples were collected from 190 tuberculin-positive animals (105 dairy cattle and 85 buffaloes), 235 tuberculin-negative animals (125 dairy cattle and 110 buffaloes) and 95 mixed market samples. Moreover, serum samples were collected from tuberculin-positive animals to be tested with ELISA.

Microscopical examination

According to Bermudez et al. (2010) milk samples were centrifuged at 6000 rpm/15 min at 4°C and 2 loopfull of the sediment were spread on a slide, defatted with alcohol for 15 min and then stained with Ziehl-Neelsen method for detection of acid and alcohol fast bacillus.

Cultural procedures

Milk samples were aseptically cultured for the presence of Mycobacteria as previously described according to the Bermudez et al. (2010). Briefly, after centrifugation of the samples at 6000 rpm/15 min at 4°C, the sediment and the creamy layers were mixed and treated with equal volume of 6% HCl for 30 min, centrifuged and the sediment was neutralized with 4% NaOH using phenol red as an indicator and cultured on glycerin or pyruvate modified Lowenstein-Jensen slants and examined after incubation at 37°C/6 to 8 weeks for growth. Smears were made from the growing colonies and stained with Ziehl-Neelsen. For identification of the isolates, optimum growth temperature, rate of growth and pigment production were first determined and further biochemical identification was done (niacin test, nitrate production test and growth on TCH media according to Quinn et al. (2002).

ELISA detection of anti-PPD antibodies

According to Cho et al. (2007) microtiter plates (Immulon II) were coated overnight at 4°C with bovine PPD at 50 µg/ml in 50 mM carbonate bicarbonate buffer (pH 9.5; 100 µl per well as calculated by checkerboard titration). The plates were washed three times with PBS-Tween, blocked with PBS containing 10% bovine serum albumin for 1.5 h. Serum was added (diluted 1: 100 in PBS-Tween as calculated by checkerboard titration), and incubated at 37°C for 1.5 h. The plates were washed with PBS-Tween and, after the addition of antiovine conjugated peroxidase (1:3000 dilution in PBS-T), incubated at 37°C for 1 h. The plates were washed with PBS-Tween. After a final washing with PBS-Tween, citric acid buffer (50 mM, pH 5; 200µl per well) containing ortho-phenylene diamine (OPD) and H2O2 (0.02%) was added to the wells. Following incubation with shaking at room temperature for 10 min., the reaction was stopped using 1 N NaOH, 100 µl/well and the optical densities (OD), [ELISA value or absorbance] were recorded using automated ELISA reader. The tested samples were considered positive of its optical densities equal to or more than the mean value of the negative control samples by more than two standard deviations.

Guinea pigs inoculation test

1 ml of sediment and the creamy layers obtained after centrifugation were mixed and injected 1/M in the thigh of the tuberculin negative guinea pigs. The animals were euthanized 6, 8 and 10 weeks later, and tested for P/M lesions and the infection was confirmed by smear examination and culture character.

DNA extraction from milk samples

Milk samples were centrifuged for 15 min (6000 rpm, 4°C). The pellet was washed twice with TES buffer (10 mM Tris HCl, 1 mM
EDTA [pH 8.0], 100 mM NaCl,) and suspended in 4 ml of lysis buffer (50 mM Tris-HCl, 50 mM EOTA [pH 8.5] 15% [w/v], 4% SDS). Lysozyme was added to a final concentration of 100 mg/ml. The mixture was incubated at 37°C for 3 h. Proteinase K was added to a final concentration of 100 mg/ml, and incubation was continued at 56°C for 2 h. The cells were then disrupted by mechanical homogenization in glass homogenizer to each sample separately and DNA extractions were completed as mentioned by Soliman et al. (2003) and Moussa et al. (2010).

DNA amplification by PCR

Three target DNA sequences were concerned in the PCR assay. The first was a 560 bp fragments specific for 16S rRNA, which is conserved in all Mycobacterium species using MB1 (5’ GTC TCT AAC ACA TGC AAG TCG 3’) and MB2 primer pairs (Noordhoek et al., 1996). The second target was a 270 base pair region of MB3 (5’ CAT GTC TTG TGG TGG AAA GCC C3’) and MB4 (5’ CTA GCT GCT TCC AGG ACC AA 3’) primer pairs nested within the first 16S rRNA and conserved only in M. tuberculosis complex (Noordhoek et al., 1996). The third target was 500 base pair region MB5 (5’ TCG TCC GGT GAT GCA AGT GCC 3’) and MB6 (5’ CGT CCG CTG ACC TCA AGA AG3’) primer pairs which is conserved only in M. bovis (Rodriguez et al., 1995). PCR amplifications were performed in a final volume of 50 ml in micro-amplification tubes (PCR tubes). The reaction mixtures consisted of 5 ml of the DNA template, 5 µl of the extracted DNA, 5 µl of the 10X PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM MgCl2, 50 mM KCl, 20 mM (NH4)2SO4), 1 µl dNTPs (40 mM), 1 ml (1U Ampli Taq DNA polymerase), 1 µl (25 pmol) from the forward and reverse primer pairs and the volume of the reaction mixture was completed to 50 ml using DW. The thermal cycler was adjusted as follows: Initial denaturation at 94°C for 5 min, followed by 35 cycles of (denaturation at 94°C for 1 min, annealing at 56°C for MB1 and MB2 primer pairs, 65°C for MB3 and MB4 primer pairs and 68°C for MB5 and MB6 primer pairs for 1 min and extension at 72°C for 1 min). Final extension was carried out at 72°C for 10 min and the PCR products were stored in the thermal cycler at 4°C until they were collected.

Agarose gel electrophoresis

According to Sambrook et al. (1989). The PCR products were visualized by agarose gel electrophoresis. 10 µl of all PCR products were mixed with 2 µl gel loading buffer 6X stock (Bromophenol blue 0.25%; Xylene cyanol 0.25% and glycerol 30%) and then loaded onto a 1.5% agarose gel containing ethidium bromide at. The gel was subjected to electrophoresis in 1X TAE (Sambrook et al., 1989) for a suitable time that allows the bromophenol blue to run 2/3 of the gel length at 120V. A 100 bp ladder (GIBCO BRL, Life Technologies, and Gent, Belgium) and HaeIII digest (FINZYMES) were inoculated in the gel as a molecular weight standard.

Analysis of data

The sensitivity and specificity of polymerase chain reaction were calculated according to Timmreck (1994) taken the bacteriological isolation as a gold standard.

RESULTS

Tuberculin test

A total of 3255 cattle and 2650 buffaloes were tested with mammalian Tuberculin (Table 1), only 105 (3.2 %) of cattle and 85 (2.9%) of buffaloes were positive.

Post mortem findings

The tuberculin positive animals were subjected to post mortem examination (Table 3) and it was found that 78.75% of the cattle and 47.6% of the buffaloes gave localized lesion in the lungs or at least one of its associated lymph nodes, while generalized tuberculosis was 9 and 3.4% in cattle and buffaloes respectively. NVL constitutes 21 and 21.25% of cattle and buffaloes, respectively.

ELISA test

ELISA utilizing bovine PPD as an antigen gave results shown in (Table 1). Tuberculin positive cattle and buffaloes that gave positive ELISA reaction in about 93.3 and 91.1%, respectively. While in those which showed generalized lesions the results were 100% for both animal species. The animals showing NVL gave relatively much higher results 70 and 64% for cattle and buffaloes respectively. The overall results were 89.5 and 83.5% for cattle and buffaloes, respectively.
Acid fast organisms were detected in 23 (4.42%) out of 520 examined milk samples stained with Ziehl–Neelsen stain. M. bovis were isolated only from 14 (2.68%) out of 520 examined milk samples, all of them were positive with direct microscopic examination. Moreover, animal inoculation revealed positive results with 13 milk samples from tuberculin positive animals. Acid fast organism was detected in 23 (4.42%) out of 520 examined milk samples. No. of slaughtered positive reactors was calculated according to the total number of examined samples. % was calculated according to the total number of examined samples. No. positive samples.

Microscopical, bacteriological examination and animal inoculation:

M. bovis was detected in 23 (4.42%) milk samples of tuberculin positive animals. Moreover, animal inoculation revealed positive results with 13 milk samples from tuberculin positive animals. Acid fast organisms were detected by bacteriological examination although it has a specificity which approaches 100%, but the slow growth of the microorganisms results in delay in its diagnosis. Ziehl-Neelsen staining of clinical specimens lack sufficient sensitivity and species specificity (Parashar et al., 2009). Serological techniques may be useful in some clinical situations but both the sensitivity and specificity of the tests are unsatisfying (Cho et al., 2007). Therefore, the purpose of the prospective study was targeted to amplify a 560 bp fragment from the extracted DNA of milk samples by MB1 and MB2 primers of the gene coding for 16S rRNA, which is conserved in all Mycobacterium spp., the second target was a 270 bp region by using MB3 and MB4 primers nested within the first 16S rRNA and species specific primers.

DISCUSSION

Tuberculosis, caused by M. bovis is emerging as the most important disease affecting cattle. Furthermore, it results in a major public health problem when transmitted to human. Due to its difficult and non specific diagnosis, M. bovis has been declared to be one of the etiologic agents causing significant economic loss in the cattle industry. Detection of M. bovis in milk samples by bacteriological examination although it has a specificity that approaches 100%, but the slow growth of the organism results in delay in its diagnosis. Ziehl-Neelsen staining of clinical specimens lack sufficient sensitivity and species specificity (Parashar et al., 2009). Serological techniques may be useful in some clinical situations but both the sensitivity and specificity of the tests are unsatisfying (Cho et al., 2007). Therefore, the purpose of the prospective study was targeted to amplify a 560 bp fragment from the extracted DNA of milk samples by MB1 and MB2 primers of the gene coding for 16S rRNA, which is conserved in all Mycobacterium spp., the second target was a 270 bp region by using MB3 and MB4 primers nested within the first 16S rRNA and species specific primers.
Table 4. Comparison between microscopical examination, bacteriological examination, animal inoculation and nested PCR of examined milk samples.

<table>
<thead>
<tr>
<th>Laboratory diagnosis</th>
<th>Examined milk samples</th>
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<tr>
<td></td>
<td>Tuberculin negative animals</td>
<td>Tuberculin positive animals</td>
<td>Mixed market samples</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Buffaloes (110)</td>
<td>Cows (125)</td>
<td>Buffaloes (85)</td>
<td>Cows (105)</td>
<td>%</td>
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<tr>
<td>Microscopical examination</td>
<td>4.42 23</td>
<td>1.15 6</td>
<td>0.58 3</td>
<td>0.77 4</td>
<td>0.77 4</td>
</tr>
<tr>
<td>Bacteriological examination (M. bovis)</td>
<td>2.68 14</td>
<td>0.38 2</td>
<td>0.38 2</td>
<td>0.38 2</td>
<td>0.58 3</td>
</tr>
<tr>
<td>Animal inoculation</td>
<td>2.31 12</td>
<td>0.19 1</td>
<td>0.58 3</td>
<td>0.77 4</td>
<td>0.77 4</td>
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<tr>
<td>PCR using MB1, MB2</td>
<td>6.35 33</td>
<td>1.34 7</td>
<td>0.77 4</td>
<td>1.15 6</td>
<td>1.15 6</td>
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<tr>
<td>PCR using MB3, MB4</td>
<td>4.23 22</td>
<td>0.38 2</td>
<td>0.38 2</td>
<td>0.77 4</td>
<td>0.77 4</td>
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<tr>
<td>PCR using MB5, MB6</td>
<td>3.06 16</td>
<td>0.38 2</td>
<td>0.38 2</td>
<td>0.77 4</td>
<td>0.77 4</td>
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Figure 1. Agarose gel electrophoresis showing amplication of 560 base pair fragments of MB1 and MB2 primers which is conserved in all Mycobacterium species.

conserved only in *M. tuberculosis* complex. The second purpose was targeted to amplify a 500 bp fragment from the extracted DNA of milk samples directly using MBS and MB6 primers of the gene coding a conserved sequence present in *M. bovis*. Milk samples collected from tuberculin positive animals (105 cows and 85 buffaloes), as well as milk samples collected from tuberculin negative animals (125 cows and 110 buffaloes) and 95 market milk samples were tested with microscopical examination, bacteriological examination and laboratory animal inoculation. Acid fast organism was observed in 23 (4.42%) out of 520 examined milk smears by microscopical examination, only 14 samples from such milk samples were positive by bacteriological examination and yielded *M. bovis*. Moreover, 13 milk samples (2.49%) only were positive while the other samples were negative by bacteriological examination and laboratory animal inoculation and this because the microscopical examination cannot differentiate between *M. bovis* and other mycobacteria "Mycobacterium other than. *M. bovis". Our results confirm the conclusion of Parashar et al. (2009) and Bermudez et al. (2010), where they concluded that Ziehl-Neelsen staining of clinical specimens lack sufficient sensitivities and specificities. To evaluate the PCR as a diagnostic method for detection of *M. bovis* in milk samples, amplification of multicopy DNA target sequence was investigated by a nested primer strategy after extraction of DNA from milk samples by
Figure 2. Agarose gel electrophoresis showing nested PCR amplification of 560 base pair fragments of MB1 and MB2 primers which is conserved in all *Mycobacterium* species and amplification of 270 base pair fragments of MB3 and MB4 of *M. tuberculosis*.

Figure 3. Agarose gel electrophoresis showing amplification of 500 base pair fragments of MB5 and MB6 primers which is conserved in *M. bovis*.

Trizole reagent, which seems to have the highest ability for purification of high molecular weight DNA from clinical samples (Soliman et al., 2003). Amplification of 560 bp fragment of 16S rRNA which is conserved in all *Mycobacterium* spp. was observed with the extracted DNA of 33 (6.35%) out of 520 examined milk samples, of them 23 (4.42%) milk samples were positive by Ziehl-Neelsen staining technique, while the other milk samples were negative which indicate the ability of PCR to detect milk samples with non visible acid fast bacilli (negative Ziehl-Neelsen stain) which indicates the lower sensibility of Ziehl-Neelsen staining technique in comparison with the PCR, as the Ziehl-Neelsen stain require a high concentration of bacterial cells in the examined samples to be detected which confirm the results of Parashar et al. (2009) and Bermudez et al. (2010), who stated that Ziehl-
Neelsen staining technique is a low sensitive test for detection of the acid-fast bacilli as it requires a high concentration of bacterial cells (10,000 bacilli / ml or greater). Amplification of 270 bp fragments nested within the first 16s rRNA and conserved only in *M. tuberculosis* complex revealed positive amplification of 22 (4.23%) samples only while the others II samples were negative. Our results indicated that PCR technique is not only sensitive but also more reliable and specific if it is compared with Ziehl-Neelsen stain as it could identify the species of Mycobacteria which could not be identified by Ziehl-Neelsen staining technique. Our results confirm the conclusion of Bermudez et al. (2010) who stated that PCR is the method of choice for diagnosis of tuberculosis in case where the suspicious is high but Ziehl-Neelsen stain is negative, but when it gives positive result PCR permits distinction between *M. tuberculosis* complex and other mycobacterium.

Amplification of 500 bp fragment using MB5 and MB6 primers which is specific for *M. bovis*, revealed positive amplification of 16 (3.06%) milk samples with lower sensitivity if it is compared with the nested PCR using MB3 and MB4 primers. But this is may be due to MB3 and MB4 primers detecting *M. tuberculosis* complex as well as *M. bovis*.

By comparing the results of PCR and that of the bacteriological examination, it was found that PCR could detect all the bacteriological positive samples which yielded *M. bovis*. In addition, it could detect 8 milk samples with negative bacteriological examination, which indicate that the PCR has a sensitivity that equal to or greater than that of the culture methods which confirm the conclusion of Sreevatsan et al. (2000); Antognoli et al. (2001) and Perez et al. (2002); they stated that PCR assay could be effectively used as a diagnostic and/or screening test for the detection of *M. bovis* in milk from herds with bovine tuberculosis as it has a higher sensitivity if it is compared with bacteriological examination, the present results also confirm the conclusion of Parashar et al. (2009) who stated that PCR could detect the small number of culture-negative samples that contain non-viable organism or sufficient-number to be detected by culture.

Conclusion

Nested polymerase chain reaction could be used for rapid and specific detection of *Mycobacterium tuberculosis* complex in dairy product after extraction of DNA by Trizole reagent, which seems to have the highest ability for purification of high molecular weight DNA from clinical samples.

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