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Full Length Research Paper

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

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The lower sensitivity and specificity, moreover the time required for reporting the results of the traditional methods make them of low value for diagnosis of *Mycobacterium bovis.* Therefore, the purpose of the prospective study was targeted to amplify a 560 bp fragment of the 16S rRNA which is conserved in all *Mycobacterium* species and 270 bp region nested within the first 16S rRNA and conserved only in *Mycobacterium tuberculosis* complex from the extracted DNA of 520 milk samples collected from tuberculin positive and negative animals as well as market milk samples during the period of 2008 to 2010. Amplification of 560 bp fragment was observed with the extracted DNA of 33 (6.35%) out of 520 examined milk samples, out of them 23 (4.42%) milk samples were positive by Ziehl-Neelsen stain. Amplification of 270 bp fragment was observed with 22 (4.23%) milk samples, while the other 11 samples were negative. Amplification of 500 bp fragment which is specific for *M. bovis* revealed positive results with 16 (3.06%) milk samples only. Polymerase chain reaction (PCR) could detect all samples yielding *M. bovis* with bacteriological examination in addition to, 8 milk samples with negative bacteriological examination. The results revealed that nested PCR could be used for rapid and specific detection of *M. 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.

Key words: *Mycobacterium bovis,* nested polymerase chain reaction, milk samples, *Mycobacterium tuberculosis* complex.

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

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animals mainly depends on clinical manifestations of the disease, skin testing, and subsequent identification of the pathogen by culturing and biochemical testing. It is known that the skin test lacks sufficient sensitivity and specificity in many cases (Huard et al., 2003). *M. bovis* may be isolated from the secretions of skin-test-negative cattle and, furthermore, these animals were not reactive to the tuberculin test (Pounder et al., 2006).

Identification of the Mycobacterium is based on the traditional method with the Ziehl-Neelsen acid-fast stain and on the pigmentation, growth rate, and gross and microscopic colony morphologies of cultures of the isolated causative organism. Biochemical methods such as tests for niacin, catalase, nitrate reduction, and urease are used to identify different species. The Ziehl-Neelsen stain is very rapid but lacks specificity and cannot be used to distinguish between the various members of the family Mycobacteriaceae (Parashar et al., 2009), while the other procedures usually require 4 to 8 weeks to obtain good growth.

Molecular techniques have been developed, most of them consisting of Polymerase chain reaction (PCR) detection, which allows species-specific diagnosis of Mycobacterial infections, these techniques greatly reduced identification time (Bermudez et al., 2010). The use of PCR has improved the level of detection in clinical specimens. It has been previously reported (Kolk et al., 1992) that by amplifying species-specific DNA sequences, and hybridizing the amplified sequence with a labeled probe, 5 µg of Mycobacteria DNA (corresponding to one mycobacterium) can be detected in clinical samples. PCR-based methods have the potential to be faster, more accurate, and the most efficient means of detecting *M. bovis*; however, PCR sensitivity has been shown to be hindered by the method used to isolate the nucleic acid target (Bermudez et al., 2010).

The use of a modification method for DNA extraction depending on the TriaZole reagent and evaluate the possible application of the PCR technique for the detection of *M. bovis* in milk from tuberculin positive and negative cattle and buffaloes as well as market milk is the major strategy of this study.

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 (l05 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 bacilli.

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 antibovine 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 H_20_2 (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 HCI, 1 mM

Table 1. Results of tuberculin test, postmortem finding and ELISA of examined animals.

Species	No. of tested animals	Positive tuberculin test		PM finding			ELISA results	
		No.	%	Site	N	%	N	*%
Cow	3255	105	3.2	L	75	78.75	70	93.3
				G	10	7.8	10	100
				NVL	20	21	14	70
				Total	105	100	94	89.5
Buffalo	2950	85	2.9	L	56	47.6	51	91.1
				G	4	11.9	4	100
				NVL	25	21.25	16	64
				Total	85	100	71	83.5

L= localized lesion; * ELISA results were calculated in relation to the site of infection; G= generalized lesion; NVL= non visible lesion; N = Number of positive samples.

EDT A [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 CTT 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 CCC 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 GCT GAT GCA AGT GC3\) and MB6 (5\ CGT CCG CGT ACC TCA AGA AG3\) primer pairs which is conserved only in *M. bovis* (Rodriquez 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 DDW. 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 I X 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 localized lesions in the post mortem 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.

Table 2. Comparison between microscopical examination, bacteriological examination and laboratory animal inoculation of examined milk samples.

Milk samples		Total number of examined samples		Laboratory diagnosis								
				Microscopical examination		Positive culture					Animal inoculation	
						M. bovis		Unidentified	Total			
				No.	%	No.	%	No.	%		No.	%
Individual milk samples	Tuberculin positive animals	Cows	105	6	1.15	5	0.96	0	0	5	5	0.96
		Buffaloes	85	4	0.77	3	0.58	0	0	3	3	0.58
	Tuberculin negative animals	Cows	125	4	0.77	2	0.38	0	0	2	2	0.38
		Buffaloes	110	3	0.55	2	0.38	1	0.19	3	1	0.19
Mixed market samples			95	6	1.15	2	0.38	1	0.19	3	2	0.38
Total			520	23	4.42	14	2.68	2	0.38	19	13	2.40

% was calculated according to the total number of examined samples. N: Number of positive samples.

Table 3. Comparison between postmortem findings, bacteriological examination and PCR of milk samples of tuberculin positive animals.

Postmortem finding	Species	No. of slaughtered positive reactors	No. of *M. bovis* +ve cultures of milk		PCR results	
			No.	%	No.	%
Localized lesions	Cows	75	1	0.95	3	2.56
	Buffaloes	56	1	1.18	1	1.18
Generalized lesions	Cows	10	4	3.81	6	5.71
	Buffaloes	4	2	2.35	2	2.35
Non visible lesions	Cows	20	0	0	1	0.95
	Buffaloes	25	0	0	1	1.18
Total	Cows	105	5	4.76	10	9.52
	Buffaloes	85	3	3.53	4	4.71

% was calculated according to the total number of examined samples. No. : Number of positive samples.

Microscopical, bacteriological examination and animal inoculation:

Acid fast organism was detected in 23 (4.42%) out of 520 examined milk smears 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 microscopical examination, while the other 9 milk samples which showed acid fast organisms with Ziehl-Neelsen were negative by bacteriological examination. Moreover, animal inoculation revealed positive results with 13 milk samples (2.49%), all of them yielded *M. bovis* with bacteriological examination as shown in Tables 2 and 4.

PCR

Amplification of 560 bp fragment of the 16s rRNA which is conserved in all Mycobacterium species 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 stain. The nested re-amplification of 270 bp fragments which is specific for *M. tuberculosis* complex using MB3 and MB4 primers revealed positive amplification with 22 (4.23%) samples only while the other 11 samples revealed negative results. Moreover, amplification of 500 bp fragment directly using MB5, MB6 primers which is specific for *M bovis,* revealed positive amplification with 16 (3.06%) milk samples as shown in Table 4, Figures 1, 2 and 3.

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 MB I 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

Table 4. Comparison between microscopical examination, bacteriological examination, animal inoculation and nested PCR of examined milk samples.

Laboratory diagnosis	Examined milk samples											
	Tuberculin negative animals				Tuberculin positive animals				Mixed market samples		Total	
	Buffaloes (110)		Cows (125)		Buffaloes (85)		Cows (105)					
	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
Microscopical examination	4.42	23	1.15	6	0.58	3	0.77	4	0.77	4	1.15	6
Bacteriological examination (*M. bovis*)	2.68	14	0.38	2	0.38	2	0.38	2	0.58	3	0.96	5
Animal inoculation	2.31	12	0	0	0.19	1	0.58	3	0.58	3	0.96	5
PCR PCR using MB1, MB2	6.35	33	1.34	7	0.77	4	1.15	6	1.15	6	1.92	10
PCR PCR using MB3, MB4	4.23	22	0.77	4	0.38	2	0.77	4	0.77	4	1.54	8
PCR PCR using MB5, MB6	3.06	16	0.38	2	0.38	2	0.38	2	0.77	4	1.15	6

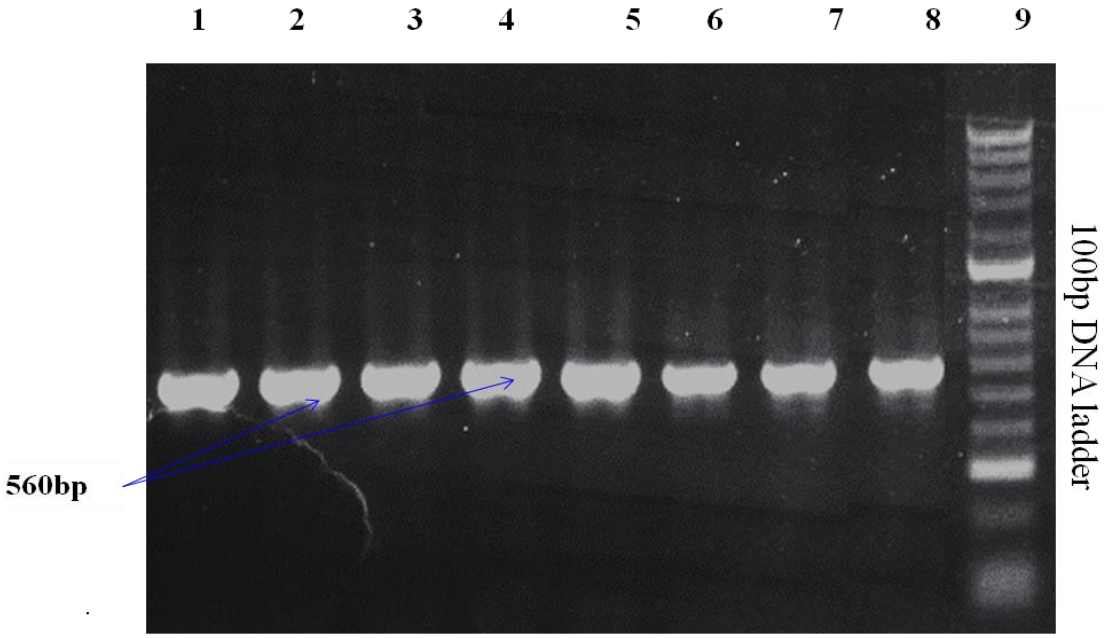

Figure 1. Agarose gel electrophoresis showing amplication of 560 base pair fragments of MB1 and MB2 primers which is conserved in all *Mycobacterium* species.

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 conservedsequence 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

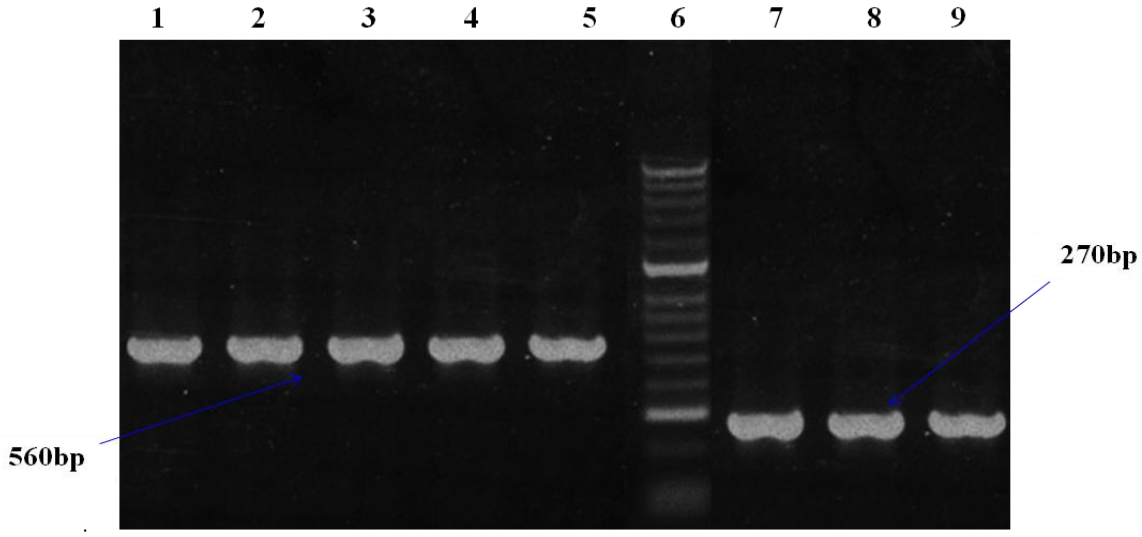

Figure 2. Agarose gel electrophoresis showing nested PCR amplification of 560 base pair fragments of MB1 and MB2 primers which is conserved in all *Mycobacterium* species and amplification of 270 base pair fragments of MB3 and MB4 of *M. tuberculosis.*

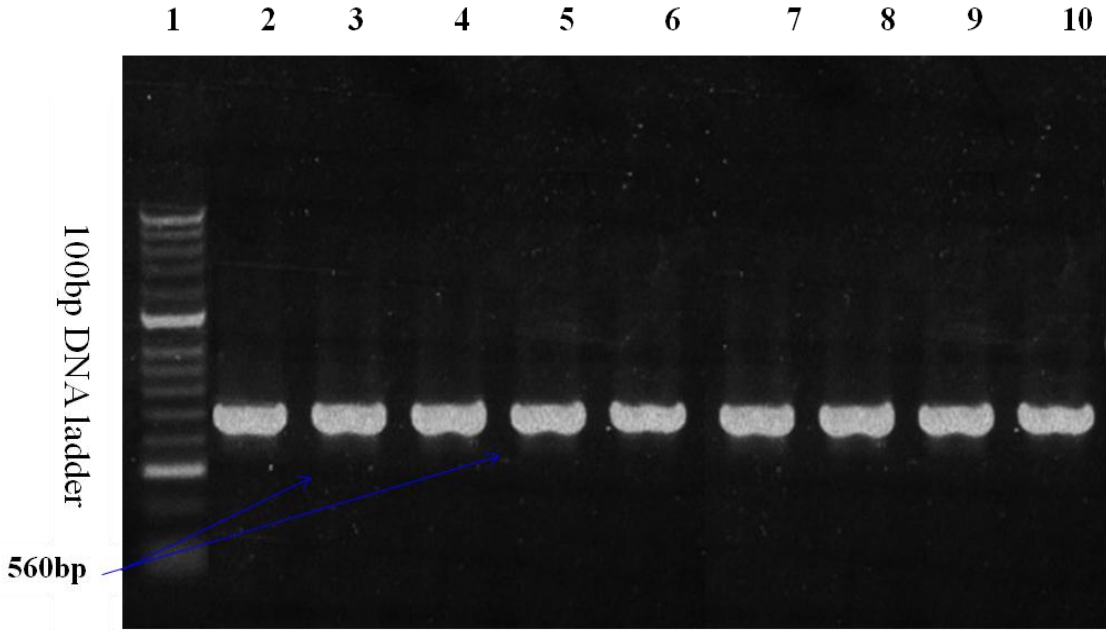

Figure 3. Agarose gel elcetrophoresis showing amplification of 500 base pair fragments of MB5 and MB6 primers which is conserved in *M. bovis.*

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 sensitivity 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 Zeihl-

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 Myobacteria 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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