COMPARISON BETWEEN THE CONVENTIONAL METHODS USED FOR DETECTION OF *M. BOVIS* IN MILK AND THE NESTED POLYMERASE CHAIN REACTION

By

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ABSTRACT

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 *M. 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 spp*. And 270 bp region nested within the first 16srRNA and conserved only in *M. tuberculosis* complex from the extracted DNA of 520 milk samples collected from tuberculin positive and negative animals as well as market milk samples. Amplification of 560 bp fragment 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. 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. PCR could detect all samples yielding *M. bovis* with bacteriological examination in addition, 8 milk samples with negative bacteriological examination. The present results concluded that PCR assay could be effectively used as a diagnostic and/or screening test for detection of *M. bovis* in milk.

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 **(Hardie and Watson, 1992)**. 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 TB- exposed animals between herds. 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. 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 live 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 **(Neill *et al.*, 1994)**. **Neill and coworkers (1992)** have reported that *M. bovis* may be isolated from the secretions of skin-test-negative cattle and, furthermore, that these animals were not anergic, as is sometimes the case in the later stages of the disease.

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, while the other procedures usually require 4 to 8 weeks to obtain good growth. In order to be certain of the diagnosis of tuberculosis postmortem histopathological examination of organ lesions is carried out. In the past few years molecular approaches to diagnosis have been transforming the investigation of tuberculosis, especially in human medicine. The introduction of PCR and nucleic acid hybridization has greatly reduced identification time **(Clarridge *et al.*, 1993)**, and 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 fg of mycobacterial 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 (e.g., RNA and DNA). For example, the solutions (e.g., NaOH) used to process mycobacterial specimens inhibit the PCR **(Kolk *et al.*, 1992)** or at least affect its sensitivity. In addition, methods involving centrifugation that

are used for preparing clinical specimens suspected of harboring mycobacteria are deficient because of the waxy cell wall (i.e., surface tension) and the buoyant nature of the mycobacteria **(Robinson and Stovall, 1941; Klein *et al.*, 1952 and Thornton, 1997)**. The difficulty associated with lysing these organisms further complicates detection. Overall, the net effect is a very limited isolation of tubercle bacilli. This is an extremely important consideration when working with samples that initially are present with low numbers of bacilli.

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

MATERIAL AND METHOD

Animals:

A total of 3255 cattle and 2650 buffaloes 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 Mackie and McCarteny (1989):

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:

Aseptically drawn milk samples were cultured for the presence of mycobacteria as previously described according to the Petroff's method. 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-

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 **Mackie and McCarteny (1989).**

ELISA detection of anti-PPD antibodies according to O'Reilly (1989):

Microtiter plates (Immulon II) were coated overnight at 4°C with bovine PPD at 50µg/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-T, blocked with PBS containing 10% bovine serum albumin for 1.5 hours. Serum was added (diluted 1:100 in PBS-T as calculated by checkerboard titration), and incubated at 37°C for 1.5 h. The plates were washed with PBS-T 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-T. After a final washing with PBS-T, citric acid buffer (50 mM, pH 5; 200 ml per well) containing Ortho-phenylene Diamine (OPD) and H_2O_2 (0.02%) was added to the wells. Following incubation with shaking at room temperature for 10 min., the reaction was stopped using 1N NaOH, 100µl/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 **(O'Reilly, 1989)**.

Guinea pigs inoculation test:

One milliliter of sediment and the creamy layers obtained after centrifugation were mixed and injected I/M in the thigh of the tuberculin negative guinea pigs. The animals were authenized 6, 8 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:

Small -scale DNA extractions from milk samples were performed as described by **Ross *et al.*, (1992); Andersen *et al.*, (1992) and Soliman, (2003)** with some modifications. Milk samples were centrifuged for 15 min (6000rpm, 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 EDTA [pH 8.5] 15% [wt/vol], 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; one ml Triazol reagent (lifetechnology) was added and incubated at room temperature for 5 minutes. After vortexing for 30 sec, 0.5 ml of chloroform was added and centrifuged for 10 min at 14000 rpm. The DNA in the interphas was precipitated with 0.5 ml of.absolute ethanol. Genomic DNA was recovered by centrifugation at 14000 rpm for 30 min, washed with 70% (vol/vol) ethanol, washed twice with 0.1M sod. citrate in absolute ethanol and finally redissolved in 50ul of 8 mM NaOH. The pH was then adjusted at 8 by adding 11,5 µl/ml of 0.1 M HEPES. Two µl of RNAase were then added and incubated at 37°C for 1 hour and further purified with wizard genomic DNA purification kit (promega). The concentration and purity of extracted DNA were calculated by readings of A_{260} and A_{280}.

DNA amplification by PCR:

Three target DNA sequences were concerned in developing the PCR assay. The first was a 560 bp region MB1 (5\- GTC CTT AAC ACA TGC AAG TCG –3\) primers of the gene coding for 16s rRNA, which is conserved in all *Mycobaterium spp.* **(Noordhoek *et al.*, 1996)**. The second target was a 270 bp region of MB3 (5\- CAT GTC TTG TGG TGG AAA GCG C-3\) and MB4 (5\- CTA GCT GCT TCC AGG CCC AA –3\) primers nested within the first 16s rRNA and conserved only in *M. tuberculosis* complex **(Noordhoek *et al.*, 1996)**. The third target was 500 bp region MB5 (5\- TCG TCC GCT GAT GCA AGT GC –3\) and MB6 (5\- CGT CCG CGT ACC TCA AGA AG –3\) primers, which is conserved only in *M. bovis* **(Rodriquez *et al.*, 1995)**. The PCR reactions were carried out in a final volume of 50µl in microamplification tubes (PCR tubes). The reaction mixture consisted of 1µl (200ng) of the extracted DNA template, 5µl 10X PCR buffer (BIOTOOLS) (75 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$), 1µl dNTPs (40µM) (BIOTOOLS), 1µl (1 U AmpliTaq DNA polymerase) (BIOTOOLS), 1µl (50 pmol) from the forward and reverse primers. The volume of the reaction mixture was completed to 50µl using DDW. 40µl paraffin oil was added and the samples were subjected to 39 cycles, each consisting of:

Initial denaturation step		94°C for 4 min
First cycle:	denaturation at	94°C for 45 sec
	Annealing at	56°Cfor (for MB1, MB2), 65°C (for MB3, MB4) and 68°C (for MB5, MB6).
	Extension at	72°C for 2 min.

Repeated for another 35 PCR cycles

Final extension at 72°C for 10 min.

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. 10µl of final PCR products were mixed with 2µl 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 concentration of 0.5 µg/ml. The gel was subjected to electrophoresis in 1X TAE **(Sambrook *et al.*, 1989)** for a suitable time that allow the bromophenol blue to run 2/3 of the gel length at 120V. A 100 bp ladder (GIBCO BRL, Life Technologies, Gent, Belgium) and HaeIII digest (FINNZYMES) were inoculated in the gel as a molecular weight standard.

RESULTS

Results of 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.

Results of post mortem findings:

The tuberculin positive animals were subjected to post mortem examination (Table 1) 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 nods, while generalized tuberculosis was 9% and 3.4 % in cattle and buffaloes respectively. NVL constitutes 21% and 21.25% of cattle and buffaloes respectively.

Results of 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 P/M 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% anf 83.5% for cattle and buffaloes respectively.

Results of 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 Table (2-4).

Results of PCR:

Amplification of 560 bp fragment of the 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 pòsitive by Ziehl-Neelsen stain. The nested reamplification of 270 bp fragment 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).

DISCUSSION

Tuberculosis, caused by *Mycobacterium 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 *Mycobacterium 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 specificities **(David, 1976)**. Serological techniques may be useful in some clinical situations but both the sensitivity and specificity of the tests are unsatisfying **(Daniel *et al.*, 1987)**. 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 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 MB5 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 can not differentiate between *M. bovis* and other mycobacteria "Mycobacterium other than *M. bovis*, MOTT". Our results confirm the conclusion of **David (1976) and Wards *et al.*, (1995)**, 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 Trizole reagent, which seems to have the highest ability for purification of high molecular weight DNA from clinical samples **(Ross *et al.*, 1992; Anderson *et al.*, 1992 and 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 Ziel-Neelsen staining technique in comparison with the PCR, as the Ziel-Neelsen stain require a high concentration of bacterial cells in the examined samples to be detected which confirm the results of **David (1976) and Wards *et al.*, (1995)**, 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 fragment 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 11 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 **Kox *et al.*, (1994)** 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 give 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 could detect *M. tuberculosis* complex not only *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 **Rodriguez *et al.*, (1995); 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 **Wards *et al.*, (1995)** 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.

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

Species	Number of tested animals	Positive tuberculin test		PM finding			ELISA results	
		No.	%	*Site	No.	%	No.	**%
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
No.= Number of positive samples.

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

Milk sample		Total number of samples examined		Lab. Diagnosis							
				Microscopical examination		Positive culture and identification				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
		Buffaloe	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
		Buffaloe	110	3	0.58	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.

No.: Number of positive samples.

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

P/M finding	Species	No. of slaughtered positive reactors	No. of M. bovis +ve culture of milk		PCR results	
			No.	%	No.	%
Localized lesions	Cows	75	1	0.95	3	2.56
	Buffaloes	56	1	1.18	1	1.18
Generalizes 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 number of examined samples.

No.: Number of positive samples.

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

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

% was calculated according to number of examined samples.

No.: Number of positive samples.

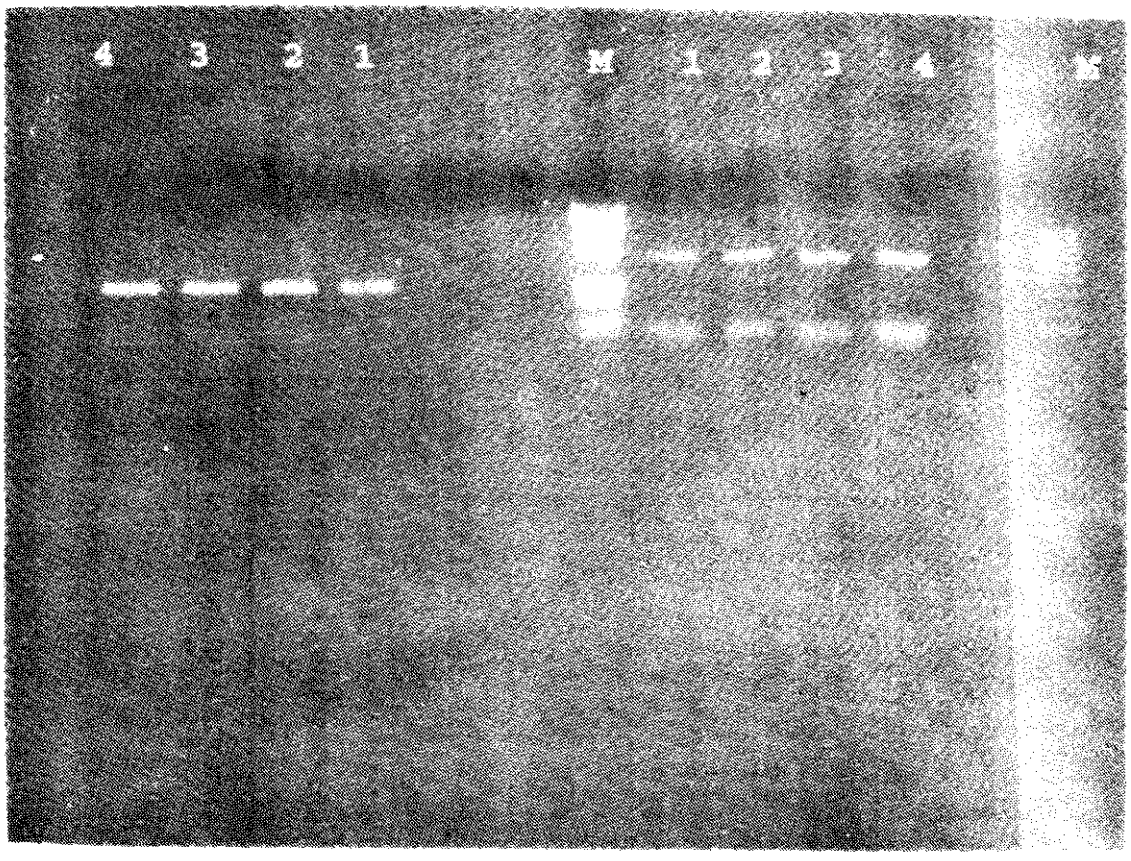

Figure (1): PCR results of milk samples using S and N primers. Note that a 500pb amplicon was found when S primer was used while on using N primer, a275 Pb band were clearly visible. 100 Pb PCR ladder was used as a marker (M).

الملخص العربي

مقارنة بين الطرق التقليدية المستخدمة في الكشف عن الميكوبكتريا البقرية في اللبن واختبار البلمرة المتسلسل المتداخل

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قلة حساسية وخصوصية الطرق التقليدية بالأضافة الي الوقت الذي تحتاجه هذه الطرق أضعف ذلك من قيمتها كطرق يمكن استخدامها لتشخيص مرض السل. لذلك استهدفت هذه الدراسة مضاعفة أجزاء طولها 560 نيكليوتيدة من الـ 16 س ر.ن.أ الريبوزومي التي توجد في كل عترات الميكوبكتريا. وكذلك مضاعفة أجزاء طولها 270 نيكليوتيدة توجد بداخل الجزء الأول الخاص بالـ 16 س ر.ن.أ الريبوزومي وهذا الجزء خاص بعترات الميكوبكتريا السلية المركبة من الحامض النووي الـ د.ن.أ المستخلص من عينات الألبان المجرعة من حيوانات ايجابية وسلبية لأختبار السلين وفي نفس الوقت عينات لبن من الأسواق. ثم مضاعفة أجزاء طولها 260 نيكليوتيدة من الـ د.ن.أ المستخلص من 33 (6.35 %) عينة كان منهم 23 عينة لبن (4.42 %) ايجابية بصبغة الزيل نيلسون. مضاعفة أجزاء طولها 270 نيكليوتيدة لوحظ مع 22 عينة فقط (4.23 %) في حين كانت العينات الـ 11 الأخري سالبة. ثم مضاعفة أجزاء طولها 500 نيكليوتيدة خاصة بالميكوبكتريا البقرية من 16 عينة لبن (3.06 %) فقط. اختبار البلمرة المتسلسل استطاع أن يكشف كل العينات التي أعطت ميكوبكتريا بقرية بالعزل البكتيري وكذلك 8 عينات كانت سلبية. أثبتت نتائج هذه الدراسة مقدرة اختبار البلمرة المتسلسل في تشخيص وكشف الميكوبكتريا السلية في اللبن.