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INCIDENCE OF TUBERCLE BACILLI IN MILK AND SOME MILK PRODUCTS IN BENI-SUEF CITY, EGYPT

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ABSTRACT

Although milk and milk products are important components of a healthy diet, if consumed unpasteurized, they also can present a health hazard due to possible contamination with pathogenic bacteria. Bovine tuberculosis (BT) is a classic example of zoonotic milk borne diseases transmitted from cattle to human. To validate this hypothesis, the current study was designed to record the prevalence of mycobacterium species in raw milk and some dairy products. Two hundred and forty two samples; (100) raw milk, (77) fresh cream and (65) kareish cheese were collected randomly from different areas in Beni-Suef city (Egypt). Mycobacterium bovis was detected in 4.95% of samples by using culturing method while the incidence by using Polymerase Chain Reaction (PCR) was 7.02% of the examined samples. These evidences reinforces the need to optimize quality programs of dairy products to intensify the sanitary inspection of these products and the necessity of further studies on the presence of mycobacterium spp. in milk and its products.

Key words: *Mycobacterium spp. Milk, Milk products, Public health.*

INTRODUCTION

Milk is the most important part of the human diet which is used in different forms as food and it is not only nature's food for a newly born infants , but also a source for a whole range of dairy products consumed by mankind. Approximately 50% of the milk produced from the dairy animals is consumed fresh, boiled or in pasteurized form. It plays a prominent role in meeting the essential human dietary requirements (protein, lactose, fat, mineral and vitamins) (Dairy Facts, 2003). The importance of the Milk intake during childhood and adolescence of dietary calcium which in the United States is derived largely from milk and dairy products to the risk of osteoporotic fractures in later life is generally assumed to be fact (Tucker, 2003).

Kareish cheese is one of soft cheeses which are most popular in Egypt and Arabian countries owing to its high protein, low fat and reasonable price. It is an acid coagulated fresh cheese, made from skim milk with soft composition white curd and slightly salty (Francois *et al.,* 2004). Kareish cheese is considered one of the most food products rich in calcium and phosphorus. These elements are essential for bones and teeth formation, it is also rich in sodium and potassium, which play an important role in the formation of body liquids and muscles (Mahmoud *et al.,* 2013).

Cream is the milk product comparatively rich in fats separated from milk either by gravity or by centrifugal separator. Microbiological analysis of cream for specific pathogens isn't considered justified and testing is restricted to potential spoilage microorganisms. (Ma and Barbano, 2000).

However, these nutrients can also serve as substrates for pathogenic microorganisms. The traditional consumption of homemade dairy products, and especially cheeses, that are composed of non-heat-treated milk pose a serious risk to public health to avoid tuberculosis (TB) infection (Di Pinto *et al*., 2006). Milk is considered a potential vehicle for transmission of some organisms which may be pathogenic for humans. External interferences in the temperature of pasteurization, extreme bacterial load contamination during milking and bottling processes may favor the survival of some species of bacteria including pathogenic or facultative pathogenic mycobacteria (Donaghy *et al.,* 2007).

Mycobacterium bovis is the etiologic agent of TB in cows and humans. Both cows and humans can serve as reservoirs. Humans can be infected by the consumption of unpasteurized milk. This route of transmission can lead to the development of extra-

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pulmonary TB. *M. bovis* affects livestock species of economic significance such as cattle (the true hosts), causing animal health problem and serious economic losses to farmers worldwide including a reduction of (10-20%) in milk production and weight (Lilenbaum *et al.,* 2001).

Mycobacterium bovis are highly able to survive in bovine milk and other dairy products. These microbes can be found in the form of viable bacilli in cream cheese and yogurt produced from raw milk for over 14 days and in butter for over 100 days (De La Rua-Domenech, 2006). Recently, the presence of these opportunistic, pathogenic bacteria in milk has emerged as a public-health concern, especially among individuals who consume raw milk and related dairy products (Chye *et al.,* 2004).

Tuberculosis (TB) is the leading cause of death in the world from a bacterial infectious disease. The disease affects 1.8 billion people/year which is equal to one-third of the entire world populations. Bovine tuberculosis is a disease of zoonotic concern, especially in countries with no control programs in livestock and where routine pasteurization of milk is not practiced (Roug *et al.,* 2014), and it was the cause of approximately 6% total human deaths due to BTB in 1930 – 1940 (Vordermeier *et al.,* 2001). It is noteworthy to mention that more than 30 million cases of TB have been registered across the world at the beginning of the 21st century (Hosek *et al.,* 2006).

M. bovis in cattle may involve in some cases udder lesions and the bacilli excreted in milk or sheded in their milk due to septicemic and cutaneous infection. In both instances sufficient levels of mycobacteria can be excreted from a single cow to make 100 gallons of previously non contaminated milk infectious for infants. Raw milk and milk products are considered the main source of primary tuberculosis in children and infants (Connell *et al.,* 2006). Tuberculosis in humans is caused by either *M. bovis* or *M. tuberculosis*. It is possible to extract surviving ancient mycobacterial DNA and amplify it by PCR. Using this technique, a laboratory performed a study of five individuals from Aymyrlyg, South Siberia, to determine the type of tuberculosis infection (Michel, 2007).

Pasteurization of milk and dairy products dropped the Bovine tuberculosis (BTB) rate drastically. Although, in developed countries, bovine tuberculosis has been considered under control, the re-emergence of the disease has been reported (Thoen *et al.,* 2006; Rowe and Donaghy, 2008).

The aim of this work was to present a credible and objective study that would be understandable to those who drink fresh unprocessed milk from our local farmers and should be afraid of becoming infected with bovine tuberculosis. So, this study was performed considering: (1) Investigation of dairy shops , markets and farmer's houses for detection of the prevalence of tubercle bacilli in collected samples;(2) Isolation of *Mycobacterium bovis* from the samples; (3)Confirmation of isolated strains of Mycobacterium Spp. by PCR; (4) Discussion of the public health hazard of the micro-organism

MATERIALS AND METHODS

Collection of samples: Two hundred and forty two samples (100 raw milk, 77 fresh cream and 65 kareish cheese samples), were collected randomly from dairy shops, markets and farmer's animals at different localities in Beni-Suef city, Egypt. All samples were transferred to the laboratory in an insulated ice box for bacteriological examination.

Preparation of samples (APHA, 1992): For milk samples, 100 ml of well mixed milk samples were centrifuged for 30 min at 3000 rpm then, freshly used or stored at -20C˚. For cream and kareich cheese samples, Ten grams of each sample were homogenized in a sterile polyethylene bag with equal volume of sterile sodium citrate 2% solution at 40˚C.

Detection of heat treated milk using Storch's test (Lampert, 1975): To 10 ml of well mixed sample which collected from dairy shops, 2 drops of diluted Hydrogen peroxide 0.2% were added before the addition of 2 drops of freshly prepared aqueous solution of paraphenylene diamine (2%). The contents were thoroughly mixed. Indigo blue color produced immediately indicates raw milk, and the light blue indicates intermediate or mixed milk, while in milk subjected to heat treatment light brown or no color is noticed.

Isolation and identification of *Mycobacterium bovis*: 100 ml of well mixed samples were transferred into sterile centrifuge tubes spun for 30 minutes at 3000 rpm. The cream and milk are poured off and the remaining sediment is mixed thoroughly with an equal volume of 6% HCL and incubated at 37˚C for 30 min. The mixture is centrifuged for 30 min at 3000 rpm., the supernatant fluid is poured off and the sediment is neutralized with 4% sterile NaOH solution using phenol red as an indicator (the change in color from orange to pink indicates correct neutralization), then, re-centrifuged for further examinations. Three to Five drops of the decontaminated sediment were inoculated in 6 screw capped bottles each two containing (Dorset egg media, Lowenstein- Jensen media and Middle brook 7H10 agar media). Inoculated tubes were sealed and incubated at 37˚C for at least 60 days with daily, and then weekly observation (Except for Middle brook 7H10 agar media was incubated for maximum 24 days) (Corner *et al.,* 1995).

The isolates were subjected to further microscopic (Kent and Kubica, 1985) and biochemical

identification (Niacin, nitrate reduction, hydrolysis of tween 80, catalase, iron uptake and urease tests), as described by (Vestal, 1975; Kent and Kubica, 1985).

Extraction of DNA (**Wards *et al.*, 1995**)

DNA extraction method for original samples: (According to thermo scientific, GeneJET Genomic purification kit #K0721, #0722). Four hundred µl of lysis solution was added and 20 µl of proteinase K to 200 µl of sample, mixed thoroughly by vortexing or pipetting to obtain a uniform suspension. The sample was incubated at 56˚C while vortexing occasionally or using a shaking water bath, rock platform or thermos mixer until the cells are completely lysed (10 min). About 200 µl of ethanol (96-100%) was added and mixed by vortexing or pipetting. The prepared lysate was transferred to Gene JET Genomic purification column inserted in a collection tube. Then centrifugation for 1 min at 6000 ×g. The collection tube was discarded containing the flow-through solution. The Gene JET Genomic purification column was placed into a new 2 ml collection tube. 500 µl of wash buffer ı (with ethanol added) was added and Centrifuged for 1 min. at 8000 ×g. The500 µl of wash buffer flow was discarded through and the purification column was placed back into the collection tube. Then 500 µl of wash buffer ǁ (with ethanol added) was added to the Gene JET Genomic purification column. Centrifugation for 3 min at maximum speed (≥ 1200 ×g). The collection tube was discarded containing the flow- through solution. The GeneJET Genomic purification column was placed into a sterile 1.5 ml microcentrifuge tube. 200 µl of Elution buffer was added to the center of the GeneJET Genomic purification column membrane to elute genomic DNA. Incubation for 2 min at room temperature and Centrifugation for 1 min at 8000 ×g. The purification column was discarded. The purified DNA was used immediately in downstream application or store at -20˚C.

Extraction of DNA from isolated microorganism: (According to thermo scientific, GeneJET Genomic purification kit #K0721, #0722). Before starting; prepare bacteria lysis buffer. Twenty mM Tris-Hcl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, add lysozyme to 20 mg/ml immediately before use. Adequate number of bacterial cells were harvested in a 1.5 or 2 ml microcentrifuge tube by centrifugation for 10 min at 5000 ×g. Discard the supernatant and suspend the pellet in 180 µl of the prepared bacterial lysis buffer. 200 µl of lysis solution was added and 20 µl of proteinase K to 200 µl of isolated microorganism, and mixed thoroughly by vortexing or pipetting to obtain a uniform suspension. The suspected microorganism was incubated at 56˚c while vortexing occasionally or using a shaking water bath, rock platform or thermo mixer until the cells are completely lysed (10min.). 20 µl of RNase A solution was added, mixed by vortexing and incubation of the mixture for 10 min. at room temperature. 400 µl of ethanol (96-100%) was added and mixed by vortexing or pipetting. The prepared lysate was transferred into Gene JET Genomic DNA purification column inserted in a collection tube. The column was centrifuged for 1 min. at 6000 ×g. The collection tube was discarded containing the flow-through solution. The Gene JET Genomic purification column was placed into a new 2ml collection tube. 500 µl of wash buffer I (with ethanol added) was added. Centrifugation for 1 min at 8000 ×g, then discarding the flow through and the purification column was placed back into the collection tube. 500 µl of wash buffer II (with ethanol added) was added to the Gene JET Genomic purification column, and centrifugation for 3 min. at maximum speed (≥ 1200 ×g), then discarding the collection tube containing flow-through solution. The Gene JET Genomic purification column was placed into a sterile 1.5 ml micro-centrifuge tube. 200 µl of Elution buffer was added to the center of the Gene JET Genomic purification column membrane to elute genomic DNA. Incubate for 2 min at room temperature and centrifuge for 1 min. at 8000 ×g. The purification column was discarded. The purified DNA was used immediately in downstream application or store at -20˚c.

Detection of M. tuberculosis complex (**Ben Kahla *et al.*, 2011)**: Real time PCR was performed according to the kit obtained from biovision® Real time PCR for samples was performed by using MTplex dtec-RT—qPCR Test designed for detection of the *Mycobacterium bovis.* Extracted DNA from the suspected samples was subjected to RT-PCR. The primers and TaqMan probe target a sequence conserved for *M. bovis*. The PCR was performed in a total of 20 µl, which contained 10 µl Hot Start-Mix qPCR 2×, 1 µl MTplex dtec-qPCR-mix, 4 µl DNase/ RNase free water and 5 µl DNA sample. The reaction conditions consisted of one cycle at 95˚C for 5 min followed by 45 cycles at 95˚C for 30 sec and 60˚C for 60 sec for hybridization, extension and data collection. the reaction was run in Applied Biosystem Step One Real Time PCR System and FAM fluorogenic signal was collected and the cycle threshold of the reactions was detected by Step One™ software version 2.2.2 (Life Technology). Sequences for primers used in this study are as follow; forwaed JB21 (TCGTCCGCTGATGCAAGTGC) and reverse JB22 (CGTCCGCTGACCTCAAGAAAG) Primer amplifying a 500 bp genomic fragment specific for *M. bovis* (Mumtaz *et al.*, 2008).

RESULTS

Results of conventional culture technique:

Table 1: Incidence of *M. bovis* in the examined samples by conventional culture technique.

Samples	No of tested samples	Positive samples	% of Positive samples
Raw milk	100	6	6.00 %
Cream	77	2	2.59 %
Kareish cheese	65	4	6.15 %
Total	242	12	4.95 %

Table 2: Incidence of *M.bovis* in the examined samples by using real time PCR assay.

Samples	No of tested samples	Positive samples	% of positive samples
Raw milk	100	8	8
Cream	77	3	3.89
Kareish cheese	65	6	9.23
Total	242	17	7.02

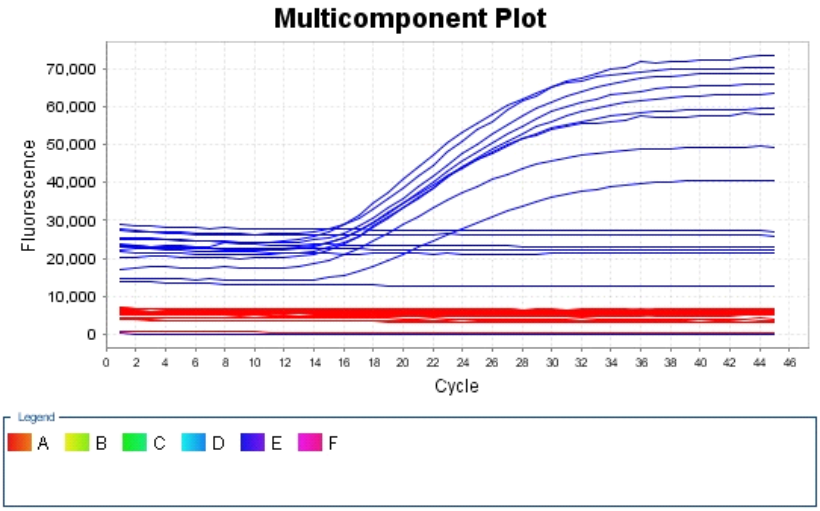

Fig.1: The amplification plot of suspected milk samples. Analysis for the amplification plot in its linear form: Eight samples at cycle 14 and one control positive sample, six negative samples. The used reference dye is (FAM). The run is for 45 cycles.

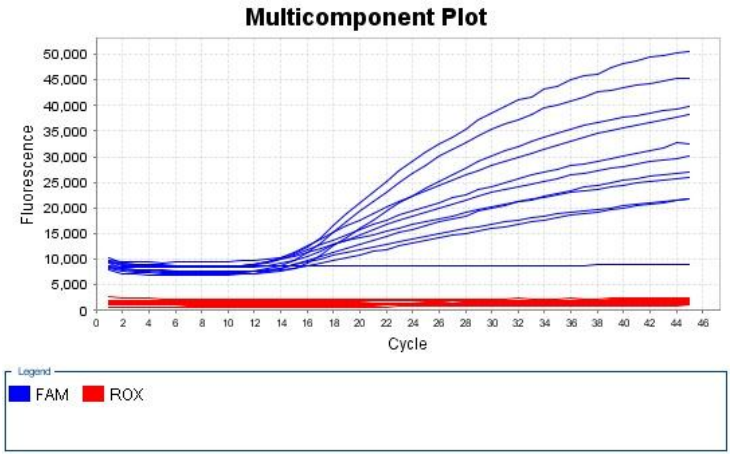

Fig.2: The amplification plot of suspected milk products samples. Analysis for the amplification plot in its linear form: Nine positive samples at cycle 14 and one control positive sample, one negative sample. The used reference dye is (FAM). The run is for 45 cycles.

Table 3: Comparison between results of culture technique and PCR assay.

Samples	No of tested samples	Culture technique		RT-PCT	
		Positive		Positive	
		No	%	No	%
Raw milk	100	6	6	8	8
Cream	77	2	2.59	3	3.89
Kareish cheese	65	4	6.15	6	9.23
Total	242	12	4.95	17	7.02

DISCUSSION

Foodborne diseases are a public health problem worldwide. The consumption of contaminated raw milk or dairy products has been recognized as a major cause of transmission of bovine tuberculosis to humans. Tuberculosis is considered one of the highest mortality rates worldwide among the infectious diseases. Eighteen people are affected with TB every minute globally and three of them die per minute (WHO, 2013). Results recorded in Table (1) revealed that out of 242 examined milk and milk products samples, 12 samples were positive for Mycobacterium bovis with a percentage of 4.95% by microscopical examination and all positive samples were harbored the acid fast bacilli. Regarding to conventional culture technique on raw milk samples (6 out of 100), Cream samples (2 out of 77) and Kareish cheese samples (4 out of 65) were positive to *M. bovis* with a percentages of 6, 2.59 and 6.15%, respectively. These findings prove that *M.* bovis is a good survivor in milk, cheese and cream (Van Brandt *et al.,* 2011).

Similar results were detected in Egypt by Wahba *et al*. (2013) who found that 6% of the examined milk samples were microscopically positive to *M. bovis* and nearly similar results were obtained by Gad *et al.* (2000) with a percentage of 5.6%.

On the other hands, lower results in Egypt, were obtained by Hassanain *et al*. (2009) who revealed that 4.35% of the collected 23 bovine milk samples were positive for *M. bovis* using culture technique, as well as Wahba *et al*. (2013) in a study conducted on market milk samples, they reported that out of 50 examined milk samples there was 1 (2%) positive by culture. In Iraq, Al- Saqur *et al*. (2009) detected 3 (4.4%) positive samples out of 68 examined milk samples by microscopical examination. In Nigeria, Ofukwu *et al.* (2008) found that 4 (1.4%) of the 285 freshly drawn milk samples were positive by culture and microscopical examination in some Makurdi markets. Additionally, lower results were obtained by Centers for Disease Control and Prevention (2005) which mentioned that investigation in New York City reported that 1% of culture-positive tuberculosis cases in milk in this area were due to *M. bovis*.

Higher results for culturing technique were determined by Abou-Eisha *et al.* (2002) in Egypt who detected that 2 (7.7%) of the 26 milk samples were positive for *M. bovis*. Also Franco *et al*. (2013) and Pardo *et al*. (2001) in Brazil were able to isolated *M. bovis* from milk samples in percentages of 8 and 10%, respectively. Furthermore, higher results were obtained by Hamid *et al.* (2003) in Pakistan who conducted a study at Lahore and isolated *M. bovis* from milk samples of four cows out of 16 (25%) with confirmed bovine tuberculosis. In Iraq, Al- Saqur *et al*. (2009) conducted a study on 68 raw milk samples and found that the positive rates for culture were 7 (10.2%).

In addition to that LoBue *et al.* (2004) in San Diego County, CA, found that 129 out of 1,931 (6.7%) samples of soft fresh cheese originated in Mexico cultured for mycobacterium were *M. bovis*. These results were nearly similar to our findings for Kareish cheese, while lower results for cheese samples were reported by Leite *et al.* (2003) who examined a total of 203 samples and proved that 10 (4.9%) were positive for bacteria belonging to the genus *Mycobacterium*, with one isolate being identified as *M. bovis.*

At the same time, De La Rua-Domenech (2006) found that microorganisms of the Mycobacterium genus, such as *Mycobacterium bovis*, are highly able to survive in bovine milk and other dairy products. The author mentioned that these microbes could be isolated from cream, cheese and yogurt produced from raw milk for over 14 days and in butter for over 100 days and mentioned that there are no validated laboratory methods that allow the certification of such untreated milk or dairy products as "free of viable mycobacteria". He found that although *M. bovis* does not multiply in milk or does so very slowly, the large number of mycobacteria that are secreted into the milk of one animal with tuberculous mastitis is generally sufficient to render the homogenized milk from 100 lactating cows.

All samples of the present study were tested and confirmed by using real time PCR. The obtained results of molecular detection of *Mycobacterium bovis* were shown in Table (2); Fig 1 & 2 revealed that, 8 out of 100 raw milk samples were positive with apercentage of 8 %.

The detection of *mycobacterium bovis* contamination in 65 kareish cheese and 77 cream samples by real time PCR revealed that 6 kareish cheese samples were positive and harbored *mycobacterium bovis* as well as 3 cream positive samples.

The use of raw milk in the production of cheese and other dairy products is considered a potential public health risk associated with tuberculous cattle, as viable mycobacteria (including *M. bovis*) have been found to survive in mature unpasteurized cheeses (Nasr *et al.,* 2014). *M. bovis* is very resistant to chemical disinfectants, including acids and alkalis, and is considered less likely than other pathogens to be affected by the pH of the cheese (Spahr and Schafroth, 2001).

Lower results were recorded by El-Gedawy *et al.* (2014) who examined one hundred bulk tank milk samples collected from three dairy farms at Sharkia Province, Egypt, for detection of *M. bovis* by PCR, and found only apercentage of 1%. Also, lower results were obtained by Silaigwana *et al.* (2012) who detected 11(5.5%) positive milk samples. On contrast, Ereqat *et al.* (2013) failed to detect any positive cases among 30 examined milk samples.

Higher results were obtained by Leite *et al.* (2003), who conduct a study on 128 bovine milk samples from retail markets in the State Sâo Paulo, out of them there were 23 (18%) positive milk samples by PCR. Also, Ogundeji *et al.* (2015) was able to isolate 9 (18%) samples as a positive to Mycobacterium out of the 50 milk samples with the PCR method. 1 (2%) out of the 9 positive milk samples was found to be *Mycobacterium tuberculosis* while the remaining 8 (16%) were detected to be *Mycobacterium bovis* after using the digestion enzyme.

Regarding to the results of Kareish cheese, Pereira-Suarez *et al.* (2014) examined 95 samples of fresh cheese that were obtained from municipal markets in the state of Hidalgo, Mexico. They reported that *M. tuberculosis* complex DNA was detected by nested-PCR amplification of a fragment of the mpb70 gene in six samples, four of which were obtained from regions with enzootic bovine tuberculosis. They concluded that cheeses prepared from raw milk and contaminated with *M. bovis* are being sold and consumed by humans, which may cause tuberculosis.

Data given in Table (3) showed the comparison between results of culture technique and PCR assay for detection of bovine tuberculosis where the results revealed that out of 242 samples only 12 samples were positive by culture technique with percentage of 4.95%, while the same samples revealed 17 positive samples by RT-PCR with a percentage reached 7.02%, Tipu *et al.* (2012) analyzed milk samples through conventional (culture) and modern (PCR) methods in dairy cattle in and around Lahore. During 10 months (May 2007 to February 2008) period, out of 1000 samples, 454 (45.4%) were positive with PCR, 69 samples (15%) were positive for direct acid fast staining and 31 samples (7%) were positive with isolation and identification of *M. bovis*. and confirmed that the presence of *M. bovis* in raw milk samples is an enormous health risk factor for milk handlers and end consumers.

Liebana *et al.* (1995) mentioned that the PCR technique is much faster than the culture methods, as it reduces the time for diagnosis from several months to 2 days. It also provides for the detection of *M. bovis* when rapidly growing *Mycobacterium* spp. are present in the sample and may be able to detect the presence of *M. bovis* in samples even when organisms have become non-viable. Additionally, Al- Saqur *et al.* (2009) proved that only three bacilli in milk samples sufficient to be detected by real time PCR. They recorded 7 (10.3%) were positive by real time PCR which are higher than that obtained by culture technique and attributed that as the PCR give. high sensitivity and specificity for the Mycobacterium This leading to the fact that real time PCR is more accurate and faster than conventional method for TB diagnosis. As early diagnosis of TB disease is crucial in initiating treatment and interrupting the strain transmission, rapid diagnosis will prevent the development of drug resistant *M. tuberculosis*.

So, the rapid detection of mycobacterium spp. is essential and important especially toward the public health issue.

CONCLUSION

Milk and Milk products is subjected to many risks of contamination by TB due to lake of TB controlling measures in farms, absence of pasteurization and sanitary methods of processing and distribution, Products from un-pasteurized cow's milk have been associated with certain infectious diseases including *M. bovis*. So, *M. bovis* can be prohibited by pasteurization of milk before consumption and during manufacturing of dairy products and culling of infected cattle herds.

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مدي تواجد ميكروب السل في اللبن وبعض منتجات الألبان في مدينة بني سويف، مصر

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على الرغم من أن اللبن ومنتجات الألبان هي عناصر هامة في اتباع نظام غذائي صحي إلا أنه قد يمثل خطورة علي الصحة العامة إذا تناوله الأنسان دون معامله حرارية نظرا لتعرضه للعديد من الملوثات البكتيريه الممرضه التي من أخطرها السل البقري الذي يعتبر من أقدم الأمراض المشتركه التي تنتقل من الحيوان للإنسان عن طريق اللبن ومنتجاته. وقد أعدت هذه الدراسه لإقرار هذه الفرضيه وذلك بالكشف عن هذا الميكروب في إجمالي عدد ٢٤٢ عينة ١٠٠ من الحليب الخام ،٧٧ من القشدة الطازجه و٦٥ من الجبنة القريش جمعت عشوائيا من مدينه بني سويف وعند فحصها بكتريولوجيا تم عزل ميكروب السل البقري باستخدام طرق الزرع علي المستنبتات المتخصصة بنسبه ٤.٩٥٪ أما بإستخدام الطرق الحديثة (تفاعل البلمرة المتسلسل) فقد تم عزل الميكروب بنسبه ٧.٠٢٪ ومن هذه النتائج يتضح لنا مدي أهميه إحتياجنا لتحسين برامج الجوده في انتاج الألبان وفحص منتجاتها وأهميه عمل دراسات مستمرة للكشف عن مدي تواجد هذا الميكروب الخطير في اللبن ومنتجاته حفاظا علي صحه المستهلك.