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# AMPLIFICATION OF A 500 BASE-PAIR FRAGMENT FROM ROUTINELY IDENTIFIED ISOLATES OF *M. BOVIS* FROM COW'S MILK IN BAGHDAD

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**ABSTRACT**
Bovine tuberculosis (BTB) caused by *Mycobacterium bovis* is a highly infectious zoonotic disease, when transmitted to humans the disease symptoms cannot be distinguished from infection caused by *Mycobacterium tuberculosis*,the agent of TB in humans. Transmission of the disease to humans is through direct contact with diseased animals and consumption of unpasteurized milk and milk products, therefore the aim of this study is to detect of these bacteria in cow's milk by routine and molecular methods. Eighty seven milk samples were taken randomly from Al Dahab Al Abyad, Al-Fudhalya and Colleges Agriculture field in Baghdad province .Conventional and molecular methods were used for the diagnosis of the disease. The results showed that two positive cases were detected by direct smear and by culturing on Lowenstein Jansen media, the results of conventional methods were confirmed by polymerase chain reaction (PCR) technique. The presence of a 500-bp fragment which amplifies a region from the genome of *Mycobacterium bovis* was evaluated by PCR on 2*M. bovis* isolates. The two isolates were PCR positive, rendering the expected 500-bp band and giving a correlation of 100% with previous microbiological characterization. These results show that this assay may be useful for diagnosis and identification of *M. bovis* in cattle.

**KEYWORDS:** Zonooses, *Mycobacterium bovis*, Cow, Milk, PCR.

## INTRODUCTION

Bovine tuberculosis (BTB) has a high incidence throughout the world, especially in developing countries. As an infectious disease, it can create important public health problems as a zoonosis (Kubica *et al*., 2003).The human populations may be infected by direct contact with diseased animals and by the consumption of non-pasteurized milk and its derivatives.BTB has been significantly widely distributed throughout the world and it has been a cause for great economic loss in animal production (Weinhaupl *et al*., 2000). *Mycobacterium bovis* is the cause of tuberculosis in cattle, and belongs to the *Mycobacterium tuberculosis* complex (MTBC) of bacterial strains (Wirth *et al*., 2008; Hershberg *et al*., 2008). The laboratory diagnosis of *M. tuberculosis* complex is currently based on acid-fast staining and cultures on solid and/or liquid media, but its sensitivity is low (Drobniewski *et al*., 1994). Cultures on solid media require up to 8 weeks of incubation to achieve the maximum Sensitivity (Kent *et al*., 1985).It is necessary to develop new diagnostic methods for bovine tuberculosis which could identify *M. bovis* directly in biological samples, such as milk or blood, without having to culture them and which would also improve the predictive value of the tuberculin test. Although the PCR has been successfully applied for the diagnosis of tuberculosis, routine application of a PCR-based method requires that the target sequence be highly specific for the microorganism and that it be present in all of the strains isolated. Rodriguez *et al*., (1995) reported a PCR assay which amplifies a 500-bp fragment from the *M. bovis* genome by using theJB21-JB22 primer pair. However, only a small number of isolateswere used in the current study, which was performed to determine whether this 500-bp fragment could be amplified from the genome of different, previously characterized. *bovis* isolates. Specific identification of *M. bovis* strains by PCR focuses on the amplification of a 500-bp DNA fragment located inside a genomic 4,999-bp region at the 3’ end of a putative gene calledRvD1-Rv2031c, which is not present in other mycobacterium species(Rodriguez *et al*.,1995).A PCR-based assay, such as the one described here, could be used to detect the presence of *M. bovis* in biological samples, such as milk, tissues, or nasal swabs, and thus become an important tool for the control and eventual eradication of the disease.

## MATERIALS AND METHODS

### Milk samples collection

Evaluation of the distribution of *M. bovis*in raw milk specimens in three geographic areas in Baghdad province (Al Dahab Al- Abyad, Al Fudhalya and Colleges Agriculture Field), eighty seven milk samples were taken randomly for this study, (45, 22, 20 respectively).Milk samples were collected aseptically in 50 ml sterile universal containers and then placed in clean cooler packs. Samples were later refrigerated till culturing.

### Laboratory work Decontaminated method using HS-SH methods (WHO, 1998)

Ten ml of the milk sample treated with 5 ml of 7% NaCl,4% NaOH and the tube mixed for 15-20 second and then incubated at 37 ºC for 20 min after this period,15 ml of phosphate buffer saline PH 6.8 was added and the tube centrifuged at 3000rpm for 15 min. The supernatant was discarded and the sediment used for preparing the smear and inoculating culture media.

**Microscopic examination**
Sediments from 87 milk samples were examined microscopically using Ziehl Neelsen staining technique for detection of acid fast bacilli (WHO, 1998).

**Cultural examination**
One drop of sediment was inoculated onto Lowenstein Jensen (LJ) slants enriched with sodium pyruvate or with glycerol. LJ slants were incubated at 37 ºC. All cultures held for 3-8weeksand inspected weekly for bacterial growth, (WHO,1998). The isolates were further identified as *M. bovis* by biochemical tests. The cells from eugonic and dysgonic colonies, that suggest the growth of mycobacteria, were examined microscopically after Ziehl-Neelsen staining for Acid-Fast Bacilli (AFB). Eugonic mycobacterium isolates were identified by conventional methods (rate of growth, colonial morphology, pigmentation, and biochemical properties) (Brasil, 1994).

**Biochemical tests**
Five biochemical tests were used in this study which include: nitrate reduction test, niacin production test, according to the procedure mentioned by the kit manufactured company (Himedia, India), Catalase test, according to the procedure (WHO, 1994) and growth on LJ slants with sodium Pyruvate encourages the growth of *M. bovis* and growth on LJ slants with glycerol favors the growth of *M. tuberculosis* complex (WHO, 1998).

**Polymerase Chain Reaction (PCR) analysis**
For mycobacterial DNA extraction, the cells from Lowenstein-Jensen medium cultures were suspended in 1 ml of distilled water in micro centrifuge tubes. DNA was liberated by boiling. The suspensions were boiled for DNA libration, for 10 min, performed according to Eisenach *et al*., (1992).JB21 and JB22Primerstargeting for the RvD1-Rv2031c region (genomic sequence) specific for *M. bovis*. These primers were prepared according to the information of Alpha DNA Company/ Montreal. PCR master mix reaction was prepared by using (Accupower PCR premix kit)* and this master mix was done according to the Bioneer company / Korea.*PCR Pre Mix it is lyophilized materials in standard PCR tube that contained all other components needed to PCR reaction such as(Taq DNA polymerase, dNTPs, Tris-HCl P H: 9.0, KCl, MgCl2, stabilizer, and tracking dye. For detection of *Mycobacterium tulberculosis* complex and *Mycobacterium bovis* by PCR, the PCR amplification mixture (20µl) which was used for detection each gene includes: master mix, include: DNA polymerase 1U;dNTPs which include:250(µm) of each d ATP,d GTP, d CTP, d TTP; 1.5 Mm of Mgcl2; 30 Mm of KCl; 10 Mm Tris-HCl (P H 9.0), 5(µl) of template DNA , 1.5( µl)of each forwarded and reversed primers and 12 (µl)of nuclease free water to complete the amplification mixture to 20 (µl),each primer (JB21 (5´-TCGTCCGCTGATGCAAGTGC-3´ and JB22 (5´-CGTGAACGTAGTCGCCTGC-3´), targeting forthe RvD1-Rv2031c sequence Amplification was carried out in a Gene Amp PCR System 9600 (Applied Biosystems), using an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denatuation at 94°C for 1 min, annealing at 68°C for 1 min, and elongation at 72°C for 1 min. Cycling was completed by a final elongation step at 72°C for 7 min. The reaction products were resolved by electrophoresis on a 1.5% agarose gel followed by ethidium bromide staining (10g/mL) and examination under UV light.

## RESULTS & DISCUSSION
*Mycobacterium bovis* is the causal agent of bovine tuberculosis (BTB), it infects approximately 50 million animals all over the world causing economic losses of approximately 3Billion dollars per year (Sechi*et al*., 1999).The disease is zoonotic, human populations may be infected by direct contact with diseased animals and by the consumption of non-pasteurized milk and its derivatives. The conventional tests have been used commonly to identify infected animals in developing countries (Gilbert *et al*., 2005). From a total 87 milk samples, one sample (1.2%) was positive for direct smear, while two samples were positive for culture and PCR, Table (1).

**TABLE1:** The results of conventional and PCR methods

| Milk samples | Direct smear | Culture | PCR |
|---|---|---|---|
| 87samples | 1 positive | 2positive | 2positive |

Microscopically examination, which is the initial step in the diagnosis of tuberculosis, when carbol fuchsin stained the smears, these were examined under light microscope (1000X), the tubercle bacilli typically appeared as straight thin red rods with blue background, the low percentage discovered by direct smear may indicate that the bacilli load is less than 5,000 to 10,000 bacilli/ml, because the specimen is required to give a positive result by AFB staining when it loads more than 5,000 to 10,000 bacilli/ml (Liehardt & Cook, 2005). Nevertheless, owing to problems of sensitivity and specificity, direct smear from the milk gave low sensitivity, which is lower than that of culture, a negative smear does not rule out infection especially in countries where the disease is highly endemic, so the direct smear has some limitations for the detection of infected cows (Payeur*etal*., 1992; AL-Jebory, 2006).

The second important step for bovine tuberculosis diagnosis is culturing on the specific medium, this method used for detection of samples with low mycobacterial loads (Grange *et al.*, 1996). The results of culturing indicated that two milk samples were positive, the appearance of colonies were typical cream colored, buff and rough colonies against the green egg based medium, within 3-8 weeks. Cultures on solid media require up to 8 weeks of incubation to achieve the maximum Sensitivity (Kent *et al*.,1985).These results were in agreement with previous observation of Al-Sadii (1999), Salaisel (2003) and AL-Attar(2010) who found that the *M. bovis* colonies were small cream-colored, buff, non-pigmented, rough with irregular edges on the agar- base medium. Then acid-fast stain was used to stain the smears which prepared from the positive cultures, the smears showed red rod bacilli vary in size and shape, according to growth conditions and age of the culture (Chauhan *et al*., 2006). Traditionally, culture followed by the biochemical tests has been used for speciation of *mycobacterium.* Culturing

give good sensitivity (AL-Jebory, 2006). The two isolates were negative for nitrate reduction test, niacin production test, catalase test. The growth appeared on LJ slant with sodium pyruvate but did not, on LJ with glycerol, table (2).

**TABLE2:** Results of Biochemical tests for the two isolates of *M. bovis*

| Biochemical Tests | Biochemical characteristics of *M.bovis* |
|---|---|
| Niacin production | - no yellow color |
| Nitrate reduction | - no red color |
| Catalase test | - no bubbles appeared |
| Growth on LJ slants with sodium | + growth on LJ with sodium pyruvate |
| Growth on LJ slants with glycerol | - no growth on LJ with glycerol |

Success level of culture can be determined by the composition of the media used for primary isolation, particularly for *M. bovis* from milk samples, which need sodium pyruvate for the *M. bovis* growth (Grange *et al.*, 1996). *M. bovis* is unable to use glycerol as a carbon source,these organisms will often fail to grow on LJ medium with glycerol (Keating *et al*., 2005). The percentage 2 (2.3%) of the isolation, which was recorded in this study was approached to the percentage recorded by Lies Durnez *et al*., (2009) who found2.5% and 10.1%, from total of 42 collected milk samples of *M. bovis* infection and atypical mycobacterioses respectively, with more *M. bovis* infection in cattle under the extensive management system and more atypical mycobacterioses in cattle under the intensive management system. While it was somewhat higher than the percentage mentioned by Grange and Yates., (1994), Collin and Grange., (1983) who found 0.5% and 1% of shedding germ of bloody origin through the milk were appeared in the infected cow with tuberculosis in both studies, but it was much lower than the percentage which was recorded by Al-Sadii, (1999) 15(14.7%) of *M. bovis* from 102 milk samples from different regions of Baghdad province, and Al-Attar,(2010), who recorded 7(10.2%) of *M. bovis* from 68 milk samples of cattle, while Ben Kahla *et al*., (2011) mentioned that they were detected, 5(4.9%) out of 102 cow included in their study, as shedders for *M. bovis* in the milk samples of cattle. The recorded percent of isolation in the current study, was out of 87 sample which were collected from small herds distributed in different locations in Baghdad, so these results were accepted with several studies which have demonstrated, that the size of the herd is a determining factor in herd breakdowns (an outbreak of BTB in a herd): bigger herds have a bigger risk of a herd breakdown (Cleaveland *et al*.,2007).

Studies demonstrated that host genetics also play a role in the susceptibility to *M. bovis*-infection (Phillips *et al*., 2002).

Results of the conventional methods for the positive isolates were confirmed by molecular methods, the PCR gives accurate positive result, DNA were obtained from previously diagnosed TB isolates and amplificated by PCR.

A reliable PCR-based diagnostic assay must have a target DNA sequence that is specific for the microorganism to be detected and that must also be present at most, if not all, isolates of the organism. The 500-bp fragment amplified by primers JB21 and JB22 fulfills the first requirement, since it is capable of discriminating *M. bovis* from related strains (Bauer feind *et al*., 1996). Chromosomal DNA samples were resolved by horizontal agarose gel electrophoresis, the amplificated DNA bands appear at 500bp showed in the Figure (1).In this study the DNA was obtained from the two *Mycobacterium bovis* isolates which were isolated from the milk, and the results of the PCR technique showed that the two isolates were positive with percentage of 100%**,** by using primers JB21 and JB22, which amplify a 500-bp fragment of *M. bovis*.

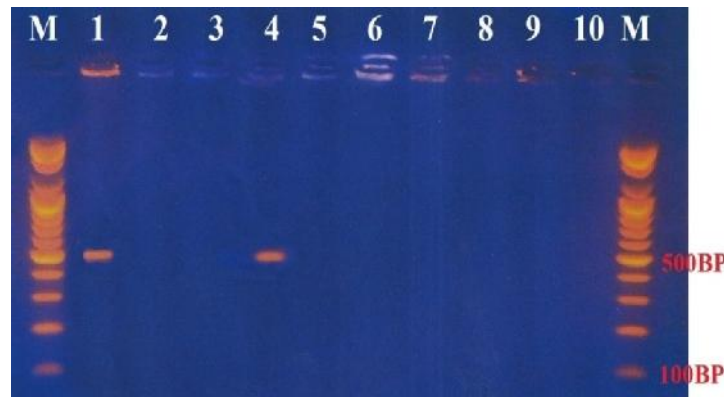


**FIGURE 1.** Agarose gel electro phoresis showed amplification of 500 bp fragments of RVD1-RV2031c genes' Marker, 1, 4 PCR amplification results of M. *bovis* appeared at 500 bp. The amplified fragments were separated by electrophoresis on a 1.5 % agarose gel stained with ethidium bromide at 60 volts for 60 min. photographed under UV light.

This fragment also fulfills other requirement that the results of this study confirmed the region amplified by primers JB21 and JB22 is conserved, since it was found in the two isolates obtained from two different regions of

Baghdad, this result was consistent with the result obtained by Rodriguez *et al*., (1995) who confirmed that the region amplified by primersJB21 and JB22 is conserved, since it was found in 121isolates from different geographic regions of Latin America.
The two isolates of the *M. bovis* strains tested by the PCR assay described here contain the 500-bp target sequence, indicating that this fragment is conserved among *M. bovis* strains, giving a correlation of 100% with previous microbiological characterization, So the PCR is a diagnostic tool for *M. bovis* detection milk as biological sample inorder to demonstrate the validity of the test for the detection of bovine tuberculosis.

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