

Detection of *Mycobacteria spp* in cows milk using conventional methods and PCR

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Abstract

Tuberculosis is a common deadly infectious disease and is important zoonotic disease. The disease can transmit to human by direct contact with the infected animals, drinking unpasteurized milk and consumption of uncooked meat. Therefore the aim of the current work is to detected of these bacteria in cow's milk. Sixty eight milk samples were taken from AL-Fthelia-Baghdad. Conventional and molecular methods were used for the diagnosis of the disease. That results showed that three positive cases in direct smear, seven positive by culturing on Lowenstein Jansen media and the result of conventional methods were confirmed by PCR, using sacace MTB Complex kit.

Keywords: Tuberculosis, Cow, Milk, PCR.

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التحري عن عصيات السل في حليب الابقار بالطرائق التقليدية وتقنية تفاعل البلمرة المتسلسل

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الخلاصة

يعد السل من الامراض المعديّة والمميّنة و من الامراض المشتركة المهمة والمسبب لهذا المرض عن جراثيم *Mycobacterium*. المرض ينتقل الى الانسان عن طريق التماس المباشر مع الحيوان المصاب وتناول الحليب الغير المبستر واللحوم الغير مطبوخة. لذا كان الهدف من هذه الدراسة هو الكشف عن هذه البكتيريا في احد منتجات الابقار وهو الحليب. تم جمع ثمانية وستين نموذج من حليب الابقار في منطقة الفضيلىة- بغداد واستخدمت الطرق الروتينية والطرائق الجزيئية في تشخيص العامل المسبب وبينت النتائج ظهور ثلاث حالات موجبة بالمسحة المباشرة وسبع حالات بالزرع الجرثومي على وسط لونشتاين جونسون وتم تأكيد التشخيص الروتيني بتقنية تفاعل البلمرة المتسلسل باستخدام عدة خاصة بمجموعة المايكوبكتريم.

Introduction

Bovine tuberculosis is an important zoonosis worldwide. *Mycobacterium bovis*, the causative agent of this disease in cattle, which has no known geographical boundaries can infect different groups of animals, which include farm animals of economic importance, wildlife, and human. *M. bovis* is a member of the *M. tuberculosis* complex, a group that includes also *M. tuberculosis*, *M. africanum* and *M. microti* (1). In Africa, approximately

85% of cattle and 82% of human population live in areas where the disease is prevalent (2). There are limited reports from India and from underdeveloped countries relating to the prevalence of and infection with *M. bovis* in cattle. Detection of *M. bovis* in bovine samples has become necessary, milk and meat are important source of protein and other nutrients but can be contaminated by pathogenic agents the possibility exists for the transmission of TB and other Mycobacterial infections from animals to humans (3). As infected animals are potentially capable of infecting

humans (zoonotic tuberculosis). Hence, *M. bovis* pose a potential health hazard to both animals and humans (4). The disease remains a major public health issue. According to the WHO, in 2004, mortality and morbidity statistics included 14.6 million chronic active cases, 8.9 million new cases, and 1.6 million deaths, mostly in developing countries with an expected 1% increase annually (5). Iraq is one of the high TB burden countries in the Eastern Mediterranean region with the highest tuberculosis burden. The estimated incidence of all TB forms accounted for 56/100,000 population in (11).

Material and methods

Evaluation of the distribution of *M.bovis* in raw milk specimens in one geographic area in Baghdad-AL-Fthelia city. 68 raw milk samples were taken for this study, the diagnosis was carried out as the following:

Decontaminated method using HS-SH methods

10ml 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 °C for 20 min after this period, phosphate buffer pH 6.8 was added and the tube centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the sediment used for preparing the smear and inoculating culture media.

The Conventional Tests include

Direct smears: prepared with 200 ul of each of the final decontaminated volumes. Smearing the specimen on the slide over an area approximately (1.5 X 1.5mm) and stained with Ziehl-Neelsen for microscopic examination (6).

Culturing: 200 ul were inoculated onto Lowenstein Jensen (LJ) slants with sodium pyruvate. LJ slants were incubated at 37 °C. All cultures held for a minimum of 3-6 weeks (6).

Biochemical tests: four biochemical tests were used in this study which include: nitrate reduction test, niacin production test, Pyrazimidase test, growth on LJ slants with sodium Pyruvate and growth on LJ slants with glycerol (6).

The Molecular Method (PCR)

DNA extraction procedure from cultures: single colonies from the culture media were suspended in 200 ul TE buffer(10 mM Tris,pH 8.0 and 1 mM EDTA) to release the bacterial DNA into the solution, the solution was heated at 95 C for 15 min (7), 100 ul of the solution used for DNA extraction by using Sacace DNA extraction kit and then the 10 ul of extracted DNA used for PCR. The Sacace MTB complex kit used for PCR, after the PCR-mix-1 tubes were prepared and then transfer them into the thermalcycler, only when temperature reached 95 C and start the program which presented in Table 1.

Table 1: Thermalcycler with block temperature adjustment: MiniCycler, PTC-100 (MJ Reaserch).

Step	C	Time	cycles
1	95 C	Pause	
2	95 C	15 min	1
3	95 C	30 sec	
	70 C	40 sec	42
4	72 C	2 min	1
5	10 C	storage	

Gel electrophoresis

2% agarose was made by adding 2 g of agarose to 100ml of 50 X TAE buffer and then it was solubilized by heating in boiling water bath. The agarose was left to cool at 50-60°C before adding the ethidium bromide and pouring the gel. 10 µl of ethidium bromide (10mg/ml) was added to the agarose.

Results and discussion

M.bovis is the causative agent of bovine tuberculosis. *M. bovis* can also jump the species barrier and cause tuberculosis in humans (1). Therefore, *M. bovis* is a relatively common cause of human tuberculosis (2).

From a total 68 milk samples, the positive rates for direct smear were three (4.4%) while the positive rates for culture were seven (10.2%) as shown in Table (2).

Table 2: The result of conventional and PCR methods.

Milk samples	Direct smear	Culture	PCR
68 samples	3 positive	7 positive	7 positive

The positive cultured on Lowenstein Jensen slant for (3-6 weeks, the appearance of colonies were typical cream-colored, buff and rough colonies against the green egg-based medium.

The isolated colonies were identified as *M.bovis* by four biochemical tests these were nitrate reduction test, niacin production test, Pyrazimidase test and the growth on (LJ) media with sodium pyruvate and the growth on (LJ) media with glycerol the results representing in Table (3).

Results of the conventional methods were confirmed by molecular methods, the PCR and it give accurate positive result DNA were obtained from all previously confirmed TB isolates and amplified by PCR. Chromosomal DNA samples were resolved by horizontal agarose gel electrophoresis, the amplified DNA bands appear at 390 bp showed in the Figure (1).

Table 3: Biochemical tests for *M.bovis*.

Biochemical Tests	Biochemical characteristics of <i>M.bovis</i>
Niacin production	- no yellow color
Nitrate reduction	- no red color
Pyrazinimadase test	- no color
Growth on LJ slants with sodium	+ growth on LJ with sodium pyruvate
Growth on LJ slants with glycerol	- no growth on LJ with glycerol

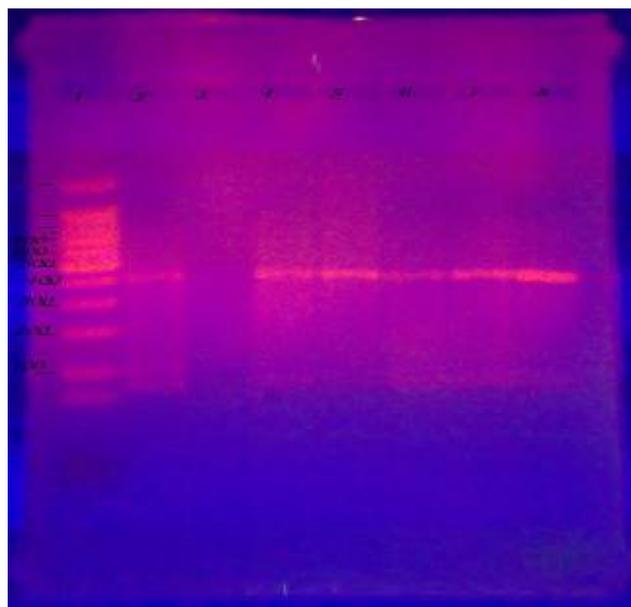


Figure (1): 1 marker, 2 positive control, 3 negative control, 4,5,6,7,8 PCR amplification results of *M.bovis* appeared at 390 bp. The amplified fragments were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide at 100 volts for 60 min. photographed under UV light.

Handling of infected cows varies in accordance with local laws in developed countries and economic compulsions and religious norms in developing countries like Africa and Asian countries. Rigorous containment and elimination of infected animals in developed countries has contributed to reduce the prevalence of bovine tuberculosis. Reliable diagnosis of bovine tuberculosis in live animals remains a major problem among veterinarians. The conventional tests have been used commonly to identify infected animals in developing countries (8). Microscopically examination which is the initial step in the diagnosis of tuberculosis. Nevertheless, owing to problems of sensitivity and specificity, direct smear from the milk gived low sensitivity. A negative smear does not rule out infection, especially in countries where the disease is highly endemic so the direct smear limited to the detection of

infected cows. Traditionally, culture followed by the biochemical tests has been used for speciation of *mycobacteria*. Culturing give good sensitivity, overall, the sensitivity of the direct acid-fast smear is lower than that of culture (10), success level of culture can be determined by (1-) growth rates and the presence of associated microorganisms which would hinder the prospect of isolation of slow growers (2-) notwithstanding prolonged incubation, the inability of potential isolates to adapt to in vitro culture conditions, especially in situations where the number of bacilli are limiting, which could result in false-negative results by culture (3-) the variability in the tedious process of identification, which has been reported to be a problem and (4-) the composition of the media used for primary isolation, particularly of *M. bovis*, from clinical isolates. The biochemical testing determined the identification of *M.bovis* in this study. However, with these conventional technique it is not possible to distinguish between *M. tuberculosis* complex and nontuberculus mycobacteria (NTM), the PCR methods can differentiate between *M. tuberculosis* complex and NTM. Although molecular techniques have been widely evaluated in diagnosis of TB. The PCR should be particularly useful for confirmed the result of the conventional methods which biochemical and growth data are difficult to obtain. The PCR give high sensitivity and specificity for the *Mycobacterium* (9). The method is more accurate and faster than conventional method for TB diagnosis. 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* bacteria (10).

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