Bovine tuberculosis: Diagnosis by PCR technique in bovine milk samples

Al-Rubiaii E.M.; Alwan M.J. and Al- Thwani A. N.
Zoonosis unit, College of Veterinary Medicine, University of Baghdad, Iraq
E-Mail: drmohammed835@yahoo.com
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Summary

The aim of this study was to determine the percentage of borne tuberculin infection in milk sample by using PCR. (102) milk samples were collected from cows **,** AL-Dejella (39) samples, AL-Suara (20) samples cow station, AL-Fthalia (20) samples, AL-Azezia (11) samples and AL-Twarege (12) samples during the period July 10th 2010 to Nov.30th 2010. The samples were examined by direct smear stained by Ziehle-Neelson stain, culture methods and confirmed the isolates by Polymerase Chain Reaction (PCR) assay. The results showed that 5 out 102 (4.9%) milk samples were *M. bovis* positive that were detected by direct milk smear method and 10 out 102(9.8%) M.bovis +ve were detected by culture method and PCR assay. The results also showed that high percentage of bacterial isolates from milk samples AL-Dejella city show (12.8%) by culturing and PCR method followed by AL-Suara (10%), AL-Fthelia (10%), Al-Twarege (8.3%) but no bacterial isolation was recorded in AL-Azezia milk samples. This study concluded that *M.bovis* infection was spreading in dairy cow within the mentioned areas and PCR was more sensitive, rapid, and accurate technique for *M.bovis* infection diagnosis.

Keywords: PCR technique, Bovine tuberculosis, Milk.

Introduction

Mycobacterium bovis is the causal agent of bovine Tuberculosis (TB), it infects approximately 50 million animals all over the world causing economic losses of approximately 3 billion dollars per year (1).

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 product derivatives, it is considered one of the leading diseases in the world and is responsible for more than 2 million deaths and 8 million new cases annually as well as one-third of the world's population latently infected (2). In development countries, bovine TB in human ranges from 0.5 to 7.2% of TB cases, while in developing countries, where very little data is available, and this figure may be as high as 15% (3). Human populations may be infected by direct contact with diseased animals and by the consumption of non-pasteurized milk and its derivatives, infected meat as well as inhalation of aerosols (4 and 5). Because of the ongoing economic and public concerns the present study aimed to determine the percentage of bovine tuberculosis infection in milk samples by PCR technique.

Materials and Methods

Milk samples were collected from Al-Dijella cows station (39) samples, Al-Suara cows station (20) samples, Al-Fthalia city (20) samples, Al-Azezia city (11) samples, and Al-Twarege city (12) samples. The decontamination of milk samples was performed according to HS-SH procedure (WHO, 1998). It took 5ml of milk sample and mixed it with 2.5ml from (7%Nacl) and 2.5ml (4%NaOH) and shacked for 20second, leave it at room temperature for 15 min. and after this neutralization with phosphate buffer saline and centrifugation at 3000 rpm for 15 min. The supernatant was carefully discarded, and sediment re-suspended in 1-2 ml of phosphate buffer pH 6.8.

The suspension was used to inoculate culture media (Stone brink media) which prepared according to (6) and to prepare smears for microscopic examination. The smears were prepared by inoculating 200µl from final decontaminated specimens on slide smears and stain with Ziehl-Neelsen's method for microscopic examination and inoculating 200µl on duplicate culturing media of stone brink media and incubated in 37C°for 6-8 weeks. During the incubation period, the slant media was observed daily. Niacin, nitrate

reduction, and pyrazimidase tests were done for positive cultures to confirm *M.bovis.*

Deoxyribonucleic acid extraction was performed by using sacacce extraction kit (Italy). Part of colony from culture media were suspended in buffer solution and heated up to 85-90C° for 10min. and this solution used for extraction of DNA by using sacacce DNA extraction kit and then 10μl from extracted DNA used in PCR reaction. Two sets of primers were used as follow:

The forward primer CSB1 (5' TTC CGA ATC CCT TGT GA3'). The reverse primer CSB2 (5' GGA GAG CGC CGT TGT A 3'). (Alpha DNA/Canada). Each μl of primer contains (35) pica moles.

PCR mixture reaction contains 3μl of template DNA, 5μl of each primers, 25μl master mix (Promega), and 12μl of distilled water free from DNase and RNase (total volume, 50μl). It optimized the PCR cycling parameters as follow: initial denaturation at 94C° for 5 minutes, followed by 30 - three step cycles, including denaturation 94C° for 1 minute, annealing at 52.3C° for 1.5min, extention at 72C° for 1 min and final extention at 72C° for 5 min.

The amplification product of DNA were analyzed by gel electrophoresis on 2% (w/v) and visualized by ethidium promide fluorescens. The unique amplification product for M.bovis would weight 168 pb.

Results and Discussion

The result showed five out 102(4.9%) milk samples were Mycobacterium +ve. The microscopic examination of the acid fast stain expressed straight red thin bacilli against blue back ground color (Table,1). The result showed buff, irregular edge white colonies embedded in the stone brink medium after 6-8 weeks post- incubation at 37^0C then acid fast stain was used to stain smear prepared from positive cultures and all these culture smears showed red thin bacilli variable in size that the bacterial isolates are *M. bovis* (Fig,1). In order to complete confirmation of the *M. bovis* isolates, all positive culturing isolates were examined by PCR assay. The result showed that all positive culture isolates were positive in PCR assay (Fig. 2), (Table, 1).

The features of *M.bovis* colonies and acid fast stained of the bacterial smear were in agreement with those described by (7). The results showed that the positive samples were variable according to area of milk samples collection and the method that used in bacterial diagnosis (table, 1). These result showed that 5 (4.9%) out 102 milk samples expressed Mycobacterium +ve by direct smear stained with Ziehle Neelsen stain while the bacterial culturing method revealed that 10 (9.8%) out 102 milk samples were *M.bovis* +ve and all these bacterial isolates gave positive result with PCR assay(table,1). Also the present study showed that the high percentage of bacterial isolates, 5(12.8%) out 39, in Al-Dyjalla station and from Al-Suara station the isolation was 2(10%) out of 20 and from Al- Fthalia city 2 (10%) of 20 and 1(10%) out of 12 samples from Twarege city, while Al- Azezea city express negative result for culturing and direct smear. The present study demonstrated that the result of direct smear-Ziehle Neelsen stain method represented the lowest percentage as compared with other methods that used in the present study (4.9%), and these results indicated that the direct smear from milk samples stain with acid-fast stain lacked sufficient sensitivity and species specifity. These evidences were in consistent with (8) who found that three positive cases of 68 samples by direct smear, also (9) showed among 520 examined milk samples 23(4.42%) milk samples were *M.bovis* positive by Ziehle –Neelsen stain, also (10) reported (4.4%) of direct smear from cattle

Milk samples showed Mycobacterium +ve. The bacterial culturing which was identified by biochemical test revealed 10% of milk samples expressed *M.bovis* +ve , these result was confirmed the result of (8) who found that seven cases *M.bovis* + ve by culturing on Lowenstein-Jensen media with pyruvate, also (8) recorded 7 isolates of *M.bovis* from 68 milk samples, in addition, in Brazil (10) noticed high percentage of *M.bovis* 23(18%) out 128 milk samples. The result of bacterial isolates in the present study was variable according to the area that milk samples were

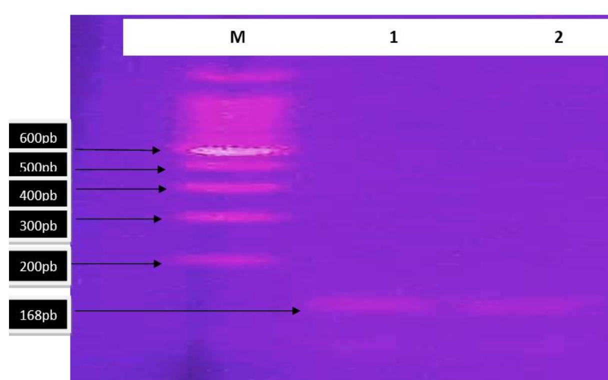
collected and examined. 102 milk samples collected from cattle apparently healthy in different areas showed that the positive results were variable according to areas that samples were collected. These differences may be due to the health care and immune state of the animals.

The present study showed that high percentage of cattle were infected with *M. bovis,* these result might be due to the lack of efficiently control program, false result of routine skin test, wild reservoir and poor nutritional condition of the animals also the infected animals shaded the microorganism to the environment , all these factors facilitated spreading the disease among animal herds and human population. However, the detection of *M. bovis* in the (10%) of milk samples of animal apparently healthy in the current study have economic and zoonotic importance in Iraq, since the infected cattle being a source of Mycobacterial pathogens capable of infecting animals and humans via direct contact between farm animals and milker's and other humans through consumption of unpasteurized infected milk as well as infected cattle would naturally facilitate a bidirectional infection of Mycobacterial pathogens therefore a more sensitive, specific, and rapid test is required for the diagnosis of tuberculosis in cattle. PCR-based methods are useful for rapid detection of target DNA in suspected clinical samples, the efficacy of PCR assays can be influenced by the quality of target DNA extracted from appropriate samples and the presence of sample-derived PCR inhibitors (11).

The results of PCR assay confirmed the results of bacteriological culturing in the present study, these result indicated that the PCR assay was faster than culture method. These result were agreement with (8) who confirmed 7 isolates of *M. bovis* from milk sample culturing by PCR and who found that the result of culturing on Lowenstein-Jensen media with Pyruvate were confirmed by PCR using Sacace MTB complex kit.

According to the present finding, we suggested that the PCR be used as a rapid and specific method for large scale screening of TB in endemic area.

Figure,1: Showing culture of Mycobacterium bovis on stone brink medium after 6-8 weeks showing characteristic feature of colonies of this microorganism, white, buff, irregular edge,



Figure, 2: Shows PCR product of *M.bovis* (168bp) on 2% agarose gel stained with Ethidium bromide (70 Volt, 45 minutes). M= Marker (1200 bp), 1= positive result, 2= positive result.

Table 1: Shows positive *m.bovis* isolates from different areas.

location	Sample number	Direct smear (%)	Culture (%)	PCR(%)
Al-Dejella city	39	3(7.6%)	5(12.8)	5(12.8)
Al-Suara city	20	1(5%)	2(10%)	2(10%)
Al-Fthalia city	20	1(5%)	2(10%)	2(10%)
Al-Azezea city	11	0.0	0.0	0.0
Al-Twarege city	12	0.0	1(8.3%)	1(8.3%)
Total	102	5(4.9%)	10(9.8%)	10(9.8%)

(0.0) –ve (Negative)

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السل البقري :تشخيص مرض السل البقري في عينات الحليب باستخدام تقنية تفاعل البلمرة الجزيئي

ازدهار محمد الربيعي[1] و محمد جويد علوان[1] وامنة نعمة الثويني

وحدة الامراض المشتركة – كلية الطب البيطري – جامعة بغداد - العراق

الخلاصة

ان الهدف من هذه الدراسة هو عزل جرثومة السل البقري من حليب الابقار بطريقة الزرع الجرثومي والمسحة المباشرة وتاكيد تشخيص العزلة بتقنية تفاعل البلمرة الجزيئي تم جمع (102) عينة حليب من محطة ابقارالدجيلة (39) والصويرة(20) ومن منطقة الفضيلية (20) والعزيزية (11) ومن منطقة طويريج (12) خلال الفترة الزمنية 2010/7/10 الى 2010 /11/30 وفحصت العينات بطريقة المسحة المباشرة باستخدام طريقة الزيل –نلسن والزرع الجرثومي على الاوساط الزرعية الخاصة وتثبيت تشخيص العزلات الموجبة باستخدام تقنية تفاعل البلمرة الجزيئي. اوضحت النتائج بان (5) من (102) عينة (4.9%) موجبة لجرثومة السل البقري شخصت بطريقة المسحة المباشرة وان (10) من (102)(9.8%)عينة موجبة لجرثومة السل البقري شخصت بواسطة الزرع الجرثومي وتاكد تشخيصها بتقنية تفاعل البلمرة الجزيئي. كذلك بينت النتائج بان اعلى نسبة للعزل الجرثومي من عينات حليب الدجيلة (12.8) شخصت بطريقة الزرع الجرثومي وتقنية تفاعل البلمرة الجزيئي وبعدها الصويرة (10%) والفضيلية (10%) وطويريج (8.3) ولم يسجل أي عزل جرثومي من عينات العزيزية. نستنتج من الدراسة الحالية بان السل البقري منتشر في ابقار الحليب من المناطق التي جمعت منها العينات وان تقنية تفاعل البلمرة الجزيئي سريعة واكثر حساسية ودقة لتشخيص جرثومة السل البقري من حليب الابقار.

الكلمات المفتاحية : تقنية PCR , السل البقري , الحليب.