

Molecular diagnosis of bovine tuberculosis in Suez Canal Area.

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Abstract

In the present study, a total of 100 tuberculin positive cattle were subjected to post mortem, bacteriological, biochemical and molecular examination. Regarding to visible lesions, out of 85 lymph nodes samples, 70 samples were positive bacteriologically with a percentage of (82.3%). While 61 lung samples were positive bacteriologically with a percentage (75.3%). Also 56 liver samples were positive bacteriologically with a percentage (80%). And 62 spleen samples were positive bacteriologically with a percentage (82.7%). Regarding to non-visible lesions, out of 15 lymph nodes samples, 8 samples were positive bacteriologically with a percentage (53.3%). While 4 lung samples were positive bacteriologically with a percentage (44.4%). Also 4 liver samples were positive bacteriologically with a percentage (57%). And 5 spleen samples were positive bacteriologically with a percentage (71.4%). All of tested isolates gave smooth colonies without pigmentation in dark or light at 37°C. Niacin production and nitrate reduction negative result, while urease test positive. Real time PCR was applied on processed tissue samples for detection of the insertion sequence **IS6110** which specific for **MTC**, the results showed high sensitivity (96%) than the traditional methods as well as a rapid accurate assay.

Keywords: Bovine tuberculosis, post mortem, real time PCR.

Introduction

M. bovis is a causative agent of bovine tuberculosis, which is the most universal pathogen among mycobacterium species and affects on all ages of many vertebrate animals including humans. (Romha *et al.*, 2013)

Bovine tuberculosis is a zoonotic disease so it considered to be socio-economic or of public health importance and characterized by the formation of granulomas (tubercles) where bacteria is localized. These granulomas are usually yellowish and caseous or calcified, they are often encapsulated. Miliary tubercles found in lymph nodes and in different organs (Opara *et al.*, 2012).

M. bovis is an extremely slow (require incubation at 37°C for 8 weeks on solid medium) which lead to failure to detect **M. bovis** because the overgrowth of other mycobacteria

(WHO, 2011).

MTC members share about 85 to 100% DNA similarity and closeness (Imaeda, 1985). Currently the differentiation between **MTC** members based mainly on phenotypic properties such as colony appearance, time of growth, and biochemical identification (Nolte and Metchock 1995) this is due to the genetic relatedness and slow grower organism, so it is difficult to differentiate between the **MTC** members.

The insertion sequence **IS6110** is an found only within the members of **MTC**, Therefore, it is considered a useful for diagnosis of **MTC** species (Thacker *et al.*, 2011).

So, This study was aimed to make comparative studies on tuberculin positive cattle by bacteriological and molecular identification of **MTC** with detecting the best method for rapid diagnosis of the bovine tuberculosis disease.

Materials and Methods

Samples:

Animals: A total of 100 tuberculin positive dairy cattle collected from different dairy cattle farms at (Ismailia governorate, Port Said governorate and Suez governorate) in Seuz Canal Area.

Tissue samples: Lymph nodes and different organs of positive tuberculin cattle at slaughter house after being slaughtered. All samples put in ice box and transfer to the laboratory for rapid isolation and identification of *Mycobacterium spp.*

Single Tuberculin Test: Was carried according to (OIE, 2009) and reported the results under supervision of veterinarian in the target farms.

Bacteriological examination: Different tissue samples which collected at slaughter house were processed and cultured into Lowenstein-Jensen medium according to (OIE, 2013). Detection of acid fast bacilli by made direct smears processed samples and stained by Zeihl- Neelsen stain according to (OIE, 2009).

Identification of *M. bovis* according to: (Kubica, 1973): Depending on morphological, growth rate, effect of light on pigment production, growth at different degree of temperature and biochemical identification including (Niacin production test, urease test, Nitrate reduction test, Hydrolyses of Tween 80, Catalase test, Iron up take, Sodium chloride 5% tolerance, Arysulfatse test and Growth on MacConkey agar).

Molecular identification of *MTC* using Real time PCR (RT-PCR) according to: (Liebana *et al.*, 1995).

A- Preparation of samples for DNA extraction

Each part of infected tissue was homogenized in PBS, pH 6.5 according to (Wards *et al.*, 1995)

B- Isolation of mycobacterial DNA from the infected tissues:

The extraction was carried out according to instruction of extraction kit of Vivates GF-1 (sigma) as follow:

- **Lysis and digestion:** 20mg of grinded tissue

+ 180µl digestion sol. + 20 µl proteinase K + mix and incubate at 56°C for 3hr.

- **Fixation:** Transfer lysate to purification column, centrifuge for 1min/ 8000 rpm, discard the collection tube then place column into new collection tube.

- **Washing:** Add 500 ul wash buffer 1, Centrifuge for 1 min./ 10000 rpm then discard flow-through, add 500 ul wash buffer 11 + centrifuge 4 min./ 14000 rpm, discard collection tube.

- **Elution:** Put column in new microfuge tube, add elution buffer + incubate 2 min. + and centrifuge for 1 min/ 10000 rpm.

C- Detection of *MTC*.

RT-PCR was done according to the data gained from biovision® company. RT-PCR was done according to Ben Kahla *et al.*, (2011) by using of MT plexdtec-RT-q PCR technique (Edifici-Quórum3, Spain) that consists of species-specific reagents prepared for diagnosis of *MTC* species.

The extracted DNA from infected tissue specimens was directed to RT- PCR. The primer and probe sequence contained all *MTC* members. The final volume of the reaction was 20 µl (comprised from 10 µl Hot Start-Mix qPCR 2x + 1 µl Target species dtec- qPCR-mix+ 4 µl DNase/ RNase free water + 5 µl extracted DNA). The conditions of the reaction contained of 1 cycle of activation (95°C for 5 min) then 45 cycles of (denaturation step : 95°C for 30 sec) and (hybridization, extension and data collection step: 60°C for 60 sec). The reaction was occur in Applied Biosystem Step One RT- PCR device and Step One™ software version 2.2.2 used for detection of the cycle threshold (CT).

Table (1). Primer used for detection of *MTC*.

Microorganism	Gene	Primer	Reference
<i>MTC</i>	<i>IS6110</i>	lxml:CGTGAGGGCATCGAGGTGGC- lxml:GCGTAGGCGTCGGTGACAAA-	Figueiredo <i>et al.</i>, (2010)

Results

Result shown in table (2), fig. (1&2) illustrated the Post mortem examination and the bacteriological examination of slaughtered tuberculin positive cattle by conventional culture method. Regarding to visible lesions, out of 85 lymph nodes samples, 70 samples were positive with percentage (82.3%). While 61 lung samples were positive with percentage (75.3%). Also 56 liver samples were positive with percentage (80%). And 62 spleen samples were positive with percentage (82.7%).

Regarding to non-visible lesions, out of 15 lymph nodes samples, 8 samples were positive with percentage (53.3%). While 4 lung samples were positive with percentage (44.4%). Also 4 liver samples were positive with percentage (57%), and 5 spleen samples were positive with percentage of (71.4%).

Table (2). Results of visible and non-visible lesions on slaughtered tuberculin positive cattle.

Types of samples	No. of tested cattle (100)		Positive culture			
			VL		NVL	
	VL	NVL	No.	%	No.	%
Lymph nodes	85	15	70	82.3	8	53.3
Lung	81	9	61	75.3	4	44.4
Liver	70	7	56	80	4	57
Spleen	75	7	62	82.7	5	71.4

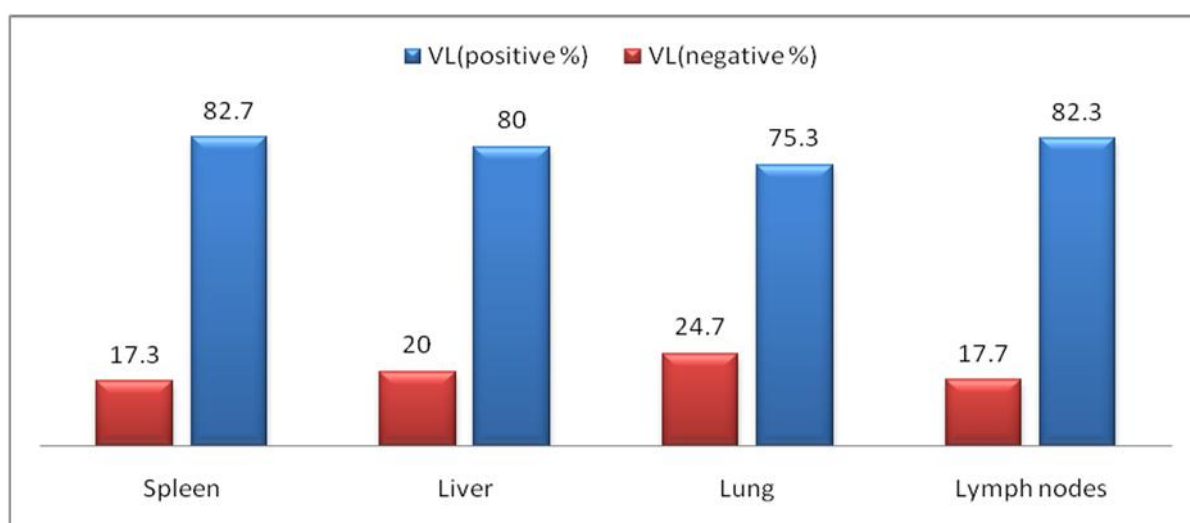
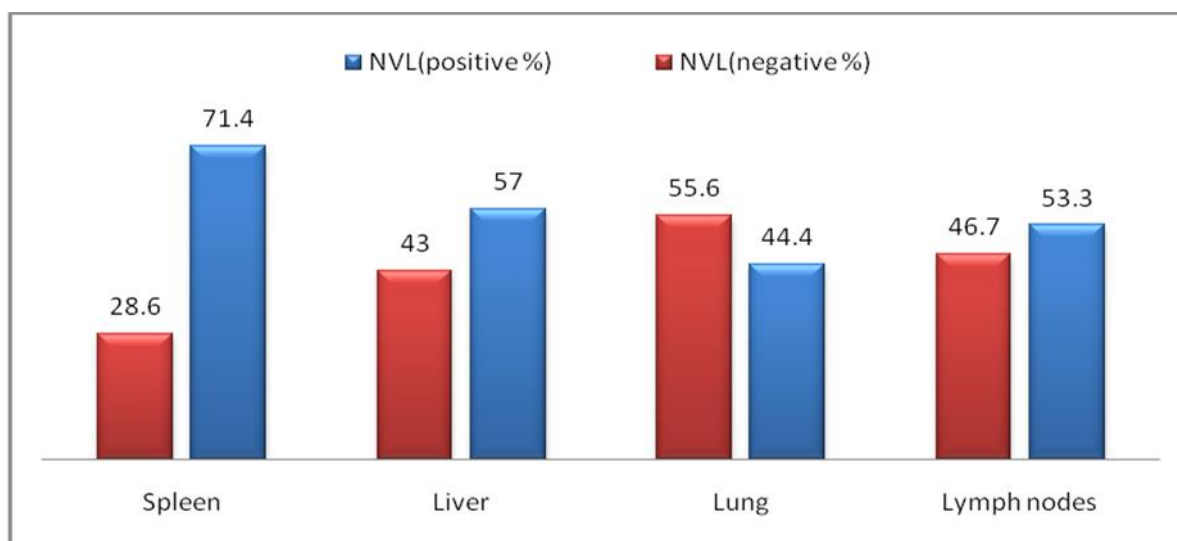
Fig. (1). Percentage of positive visible lesions by culture technique.

Fig. (2). Percentage of positive non-visible lesions by culture technique.

Results shown in table (3) revealed the physical and biochemical identification of 78 *M. bovis* isolates. All of these isolates gave

smooth colonies without pigmentation in dark or light at 37°C. Niacin production and nitrate reduction negative, while urease test positive.

Table (3). Results of physical and biochemical identification of the isolated acid fast bacilli from suspected lesions.

Test or Property	<i>M.bovis</i>
Number of isolates	78
Growth at 37°C	Slow
Colony morphology	Smooth
Dark pigmentation	–
Photo pigmentation	–
Niacin production	–
Nitrate reduction	–
Tween 80 hydrolysis	–
Catalase semiquantitative < 45°C	–
Catalase PH 7-68°C	–
Aryl sulfatase (3 days)	–
Aryl sulfatase (3 weeks)	–
Urease	+
Iron uptake	–
Growth on T2H	–
Growth on NaCl 5%	–
Growth on Macconkey agar	–

The results shown in table (4) revealed that by using of real time PCR technique, out of 100 processed tissue samples from tuberculin posi-

tive cattle, 96 tissue samples were positive for detection of *IS6110* sequence with percentage of (96%).

Table (4). Results of the real time PCR on the infected tissues and Mycobacterium isolates

Type of samples	No. of tested samples	RT- PCR		Un identified slow grower	
		No.	%	No.	%
Tissue samples	100	96	96	4	4

Photo (1): Real time PCR on tissue samples of tuberculin positive cattle for detection of the insertion sequence *IS6110* which comprises a series of species-specific targeted reagents designed for detection of all species contained in the *MTC*.

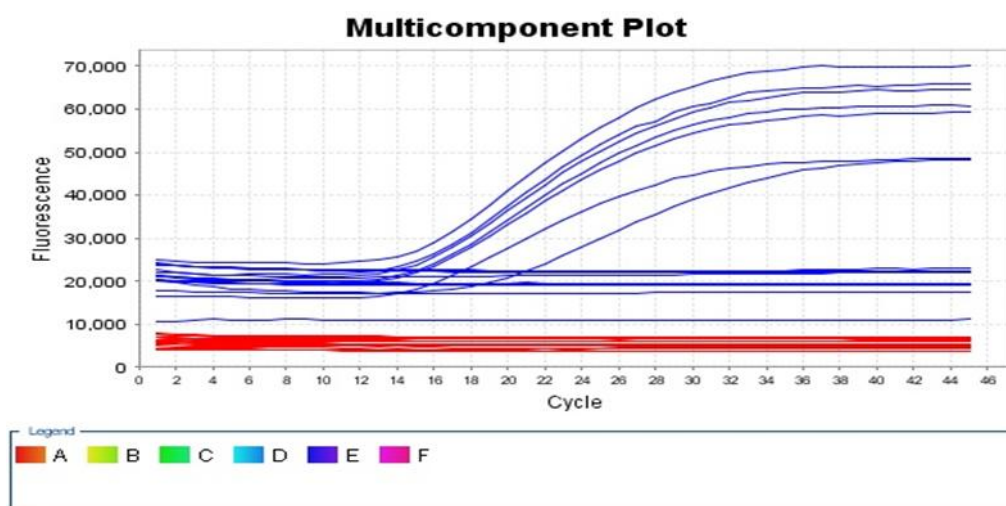


Photo (1): Results of real time PCR for detection of *MTC*.

This photo consisted of 6 positive samples at cycle 14 and one control positive sample.

There are 1 Control negative

1 Control +ve of (MOTT) Microorganism other than tuberculosis

2 Negative samples.

3- The used reference dye is (FAM).

4-The run is for 45 cycles.

Discussion

Bovine tuberculosis is a zoonotic disease so it considered to be socio-economic or of public health importance and characterized by the formation of granulomas (tubercles) in lymph nodes and in different organs and affects on all ages of many vertebrate animals including humans. (Opara *et al.*, 2012).

The obtained results in table (2) and Fig (1&2) declared that the post-mortem examination 82.3% showed VL, while 53.3% showed NVL. These results lower than obtained by (Guindi *et al.*, 1980) (VL 100%) and higher than ob-

tained by (Ereny, 2011) (VL 69.4% & NVL 30.6%). The high percentage of VL indicates that most cattle were in a chronic stage of infection. While the result of tuberculin positive reactors with NVL due to non-specific reaction by a typical mycobacteria or closely related microorganisms as *Nocardia* or combination of liver fluke with saprophytic mycobacteria and the recovery rate from NVL reactors may be due to presence of great number of cattle during the early phase of infection (Borna *et al.*, 2009).

The recorded data illustrated that the sensitiv-

ity of microscopical examination and conventional culture technique is very low but still remains the cornerstone for the diagnosis of tuberculosis infection, especially in the samples containing low number of organisms. And there are many limitations to the tuberculin test. The first limitation is the false negative results and lowering the sensitivity of tuberculin test due to delayed hypersensitivity to the antigen, as in newly infected cattle not respond to the tuberculin test, as reactions in most animals need between 3-6 weeks post infection. The second limitation is the false positive results, as the tuberculin test cannot distinguish if the response due to exposure to *M. bovis* or due to other Microorganisms as (*Corynebacterium*, *Fasciola hepatica* and some *Nocardia spp*) (Hope *et al.*, 2005).

So there is a need for rapid sensitive and accurate methods for detection of these organisms in clinical specimens. A variety of recent techniques have been developed to reduce the time to detect and identify the mycobacteria in clinical specimens (Rishi *et al.*, 2007).

The results shown in table (4) and photo (1) revealed that detection of *IS6110* gene which comprises a series of species-specific targeted reagents designed for detection of all species contained in the *MTC*. Out of 100 processed tissue sample of tuberculin positive animals, 96 tissue samples were positive for detection of *IS6110* gene with percentage (96%). These results agree with that obtained by (Ereny, 2011 and Riad *et al.*, 2014).

MTC members share about 85 to 100% DNA similarity and closeness (Imaeda, 1985). Currently the differentiation between *MTC* members based mainly on phenotypic properties such as colony appearance, time of growth, and biochemical identification (Nolte and Metc-hock 1995) this is due to the genetic relatedness and slow grower organism, so it is difficult to differentiate between the *MTC* members.

The obtained results in this study shown that the PCR test is more sensitive than conventional culture technique and direct stained smear. The high sensitivity in addition to rapid

detection of *MTC* makes this a useful technique for detection of *MTC* and studying the diversity of isolated strains. As the PCR able to detect some of negative culture samples that contained no microscopically acid fast bacilli and it is also contained non-viable bacilli or inadequate numbers to be shown by culture technique. In addition to, the culture method need up to 8 weeks while real time PCR method only takes hours meaning that real time PCR is more faster and reliable. These results agree with (Liebana *et al.*, 1995 and Bezos *et al.*, 2014).

The false negative results of PCR technique due to few numbers of mycobacterium organisms especially in *M. bovis* infections in the containing samples and not distributed well inside the tissues so obtained low copy number of the required sequence. In addition to presence of PCR inhibitors which decrease the efficiency of PCR, inefficient extraction of DNA from samples and cross-contamination or may be due to DNA degeneration due to decontamination of specimens (Riad *et al.*, 2014).

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التشخيص الجزيئي للسل البقري في منطقة قناة السويس

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الملخص العربي

في هذه الدراسة، الكشف عن ١٠٠ حالة من الماشية الإيجابية للسلو تم فحصها بالمجازر ثم فحص العينات بكتريولوجيا وبيوكيميائيا وبالطرق الجزيئية . وعند استخدام طرق الزرع التقليدية وجد أنه من أصل ٨٥ عينة من الغدد الليمفاوية كانت ٧٠ عينة إيجابية أثناء الزرع بنسبة ٨٢.٣٪. في حين كانت ٦١ عينة من الرئة إيجابية الزرع أيضا بنسبة ٧٥.٣٪. كما كانت ٥٦ عينة من الكبد إيجابية الزرع بنسبة ٨٠٪. وكانت ٦٢ عينة من الطحال إيجابية الزرع بنسبة ٨٢.٧٪. وفيما يتعلق بالأعراض الغير ظاهره، وجد أنه من أصل ١٥ عينة من الغدد الليمفاوية كانت ٨ عينات إيجابية الزرع بنسبة ٥٣.٣٪. في حين أن ٤ عينات فقط من الرئة إيجابية الزرع بنسبة ٤٤.٤٪. كما كانت ٤ عينات من الكبد إيجابية الزرع بنسبة ٥٧٪. وكانت عدد ٥ عينات من الطحال إيجابية الزرع بنسبة ٧١.٤٪. وكانت المعزولات المختبرة أعطت مستعمرات دون أن تفرز صبغات في الظلام أو الضوء عند ٣٧ درجة مئوية. وكانت المعزولات لا تنتج النياسين ولا تختزل لانتترات في حين أن اختبار اليورياز كان إيجابي. وباستخدام التشخيص الجزيئي عن طريق تطبيق تفاعل عديد البلمرة المتسلسل ذو الوقت الحقيقي على عينات من الأنسجة للكشف عن تسلسل IS6110 المحدد للعائلة المتفطرة البكتيرية، أظهرت النتائج الحساسية العالية (٩٦٪) للاختبار مقارنة بالأساليب التقليدية واثبتت النتائج حساسية ودقة اختبار البلمرة المتسلسل في الكشف عن مرض السل في المزارع بطريقة سريعة واكيدة في التشخيص.