Veterinary Medical Journal – Giza
Faculty of Veterinary Medicine, Cairo University
(ISSN 1110 – 1423)
Accredited from National Authority for Quality Assurance and Accreditation
Giza, 12211 - Egypt

Prevalence Of Mycobacterium Tuberculosis In Imported Cattle Offals And Its Lymph Nodes

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Abstract

Abattoirs considered as sources of valuable information on the incidence of animal diseases and conditions, some of which may be zoonotic, as bovine tuberculosis which still remains a major health problem.

Effort to improve food safety is going on all the time, regarding to the strategy of slaughtering cattle. Tuberculosis (TB) is an important zoonotic disease. In this study , a total of 175 (115 samples of prescapular, prefemoral, axillary and poplitial lymph nodes and 20 liver and hepatic lymph nodes samples ,20 kidney and renal lymph nodes samples and 20 spleen samples) were collected from some Egyptian abattoirs in Aswan and Red Sea governorates from imported slaughtered animals.

The obtained results showed that, the total isolates, all of them were from internal organs and lymph nodes, and all of them were identified and the results of microscopical examination of different organs collected from tuberculin positive animals by Z.N stain revealed that 115 out of 175 examined samples showed acid fast bacilli. The lymph nodes had significantly higher number of positive results concerning to culture technique which revealed the isolation of mycobacteria from 137 positive samples out of 175 examined samples from the selected governorates in percentage of isolation reached to 65.7 % and 78.3 % respectively. The results of real time PCR technique revealed that, all of tested tissue samples of 175 tuberculin positive animals were positive with percentage (100%). These results proved the accuracy of the judgment policy of the Egyptian organization which able to eliminate all the positive cases of bovine tuberculosis in some examined governorates.

Key words : bovine tuberculosis, imported slaughtered animals, diagnosis ,

Introduction

Egyptian population increased day by day to be reached in 2016 to 92 million so the needs of Egyptian is increased from the animal products but the main source to it is the importation from many countries such as: Brazil, USA, Australia, Newzeland, Canada, south Africa and Argentine either frozen boneless meat or live animals for slaughtering to cover the gap of demand and production. **(GOVS, 2016).**

Effort to improve food safety is going on all the time, but there has in addition been a major overhaul in the last couple of years. There is no such thing as zero risk, but the EU does its utmost, through a comprehensive food safety strategy, to keep risks to a minimum with the help of modern food and hygiene standards drawn up to reflect the most advanced scientific knowledge.**(European Union,2004)**

Physical, chemical and biological agents transmitted by foods cause more than 250 known diseases in humans worldwide. Of these agents, biological infectious agents are the most significant, causing the majority of foodborne diseases (OSHA, 2015; Tango, Khan, Park, & Oh, 2016). Foodborne pathogens are associated with food processing plants and slaughtered animals, the basic raw materials of the food industry. With the exception of foods that are thermally treated to the degree of sterilization, all food products are frequently associated with microorganisms (Pattanayaiying, Aran, & Cutter, 2015).The most important risk of these infectious diseases is Bovine tuberculosis which is now generally perceived to represent the greatest threat to cattle health, its caused by M.bovis and can affect a large number of species, including humans (Cobner, 2003).

Tuberculosis (TB) is an important zoonotic disease caused by an intracellular acid-fast organism Mycobacterium sp. It has been recognized from 176 countries as one of the important bovine diseases causing great economic loss **(Hines et al., 1995; Martin et al., 1994; Samad, 2000).** TB is a contagious disease, which can affect most warm-blooded animals, including human being **(Radostits et al., 2000).**

Cattle, goats and pigs are the domestic species most susceptible to infection, while horses are relatively resistant to infection. In cattle, exposure to this organism can result in a chronic disease that jeopardizes animal welfare and productivity and in some countries leads to significant economic losses by causing
ill health and mortality. Moreover, human TB of animal origin caused by *M. bovis* is becoming increasingly evident in developing countries ***(Prasad et al., 2005).*** It is also of great economic and sanitary importance in developing countries.

In Egypt, bovine tuberculosis is an enzootic disease in spite of efforts done to control the disease. So, there is a need for application of control programs based on the use of accurate diagnostics (Zinsstag et al., 2006).Officially, control of bovine tuberculosis is based on tuberculin test and slaughter policy by application of cervical intradermal which is an approved OIE test for international trade. ***(OIE, 2009).***

In Egypt prevalence of bovine tuberculosis in cattle was high in certain Governorates such as Alexandria 6%, Dakahlia and Behera 9.6% and 14.06 % respectively during 1992.on the other hand ,the recent documented results not allowed yet. So, the aim of this study is directed to screening the rate of bovine tuberculosis in imported slaughtered cattle in some Egyptian abattoirs (in Aswan and Red Sea governorates) and also have an answer of that question: **How to protect the people of the developing countries from the diseases cannot be diagnosed or detected by their facilities without banning or suspension the importation?**

Materials & methods

Collection of Samples:

A total of 175 (115 samples of prescapular, prefemoral, axillary and poplitial lymph nodes and 20 liver and hepatic lymph nodes samples ,20 kidney and renal lymph nodes samples and 20 spleen samples) were collected from some Egyptian abattoirs in Aswan and Red Sea governorates from imported slaughtered cattle during the period of Feb.2016-Oct.2016. The samples were kept in sterile plastic bags and transferred to the laboratory without undue delay in an ice box.

(Table A) The types and numbers of collected and examined samples

Gov.	samples		Total
	Types	No.	
Aswan	prescapular, prefemoral, axillary and poplitial lymph nodes	70	100
	• Liver and hepatic lymph node	10	
	• Kidney and renal lymph nodes	10	
	• Spleen	10	
Red Sea	prescapular, prefemoral, axillary and poplitial lymph nodes	45	75
	• Liver and hepatic lymph node	10	
	• Kidney and renal lymph nodes	10	
	• Spleen	10	
Total			175

Preparation of tissue samples (Marks, 1972):

Tissues of organs and lymph nodes showed the gross lesions were shopped into small pieces under aseptic condition and the fat was trimmed in sterile mortar containing sterile sand. The trimmed tissues were crushed by the sand until they become pasty .Two ml of sterile distilled water were then added and crushing was completed till the sample became a suspension. Then, 2ml of 4% conc.H_2SO_4were added and incubated for 30 min. then diluted in 16 ml of sterile distilled water and centrifuged at 3000 rpm for 20 m^{in}. The supernatant was decanted into 5% phenol and the sediment was used for direct smear and inoculated into 4 ml of L-J medium slant then incubated at 37°C. Cultures were examined daily for one week and then once weekly for 6-8 weeks.

Identification of isolated Mycobacteria:

Physico-chemical characters: it was carried out according to (Kubica, 1973)

Morphological characters:

Smears from suspected colonies were prepared and be allowed to dry and heat fixed. The fixed smears were stained with Z.N stain and examined under oil immersion objective lens to detect the colour, shape, size, and arrangement.

Molecular Detection of Mycobacterium tuberculosis complex

The infected samples were reexamined by RT-PCR as follow:

Preparation of the samples for DNA extraction:

Each piece of infected tissue was homogenized in phosphate buffer saline (PBS) (0.14M NaCl, 4mM KCl, 8mM Na2HPO4, 2mM KH2PO4, pH 6.5 buffer according to **(Wards et al., 1995).**

Extraction of mycobacterial DNA from infected tissues:

The extraction was carried out according to instruction of extraction kit of (Sigma) **as follow:**

1. Lysis and digestion: 20mg of grinded tissue + 180ul digestion sol. + 20 ul proteinase K + mix and incubate at 56 °c for 3hr.

2. Fixation: Transfering lysate to purification column, was centrifuged for 1min. / 8000 rpm, discarded tube then place column into new collection tube.

3. Washing: was added 500 ul wash buffer 1 , was centrifuged for 1 min./ 10000 rpm then was discard flow- through, add 500 ul wash buffer 11 + was centrifuged 4 min./ 14000 rpm, discard collection tube. Elution: was Putten column in new microfuge tube, was added elution buffer + was incubated 2 min. + and was centrifuged for 1 min./ 10000 rpm.

Detection of M. tuberculosis complex:

Real time PCR was performed according to the kit obtained from biovision®

The oligonucleotide primer used to detect the Mycobacterium bovis Forward 5'-CAGGGATCCACCATGTTCTTAGCGGGT TG-3'.

Reverse 5'-TGGCGAATTCTTACTGTGCCGGGGG -3'. **(Xiu-yunet al., 2006)** Real-time PCR was performed according to **Ben Kahla et al., (2011)** by using MTplexdtec-RT-qPCR Test (Edifici-Quórum3, Spain) that comprises a series of species-specific targeted reagents designed for detection of all species contained in the **Mycobacterium tuberculosis complex (Van et al, 1991.)**

Extracted DNA from the suspected samples was subjected to RT- PCR.The primers and TaqMan probe target a sequence conserved for all strains belonging to Mycobacterium tuberculosis complex. The reaction of 20 µl final volume consisted of 10 µl Hot Start-Mix q PCR 2x, 1 µl MTplexdtec-qPCR-mix, 4 µl DNase/RNase free water and 5 µl DNA sample.The reaction conditions consisted of one cycle of 95°C for 5 min followed by 45 cycles of 95°C for 0.5 m and 60°c for 1m 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).The threshold cycle (TC) was defined as 10 times the standard deviation of the mean baseline fluorescence emission calculated for PCR cycles 3–15. For a sample to be considered positive, the corresponding amplification curve had to exhibit three distinct phases (geometric, linear, and plateau) that characterize the progression of the PCR reaction.

Results and discussion

Bovine tuberculosis is now generally perceived to represent the greatest threat to cattle health; it is caused by M.bovis and can affect a large number of species, including humans **(Cobner, 2003).** It is also of great economic and sanitary importance and developing countries.

In Egypt, bovine tuberculosis is an enzootic disease in spite of efforts done to control the disease. So, there is a need for application of control programs based on the use of accurate diagnostics. Officially, control of bovine tuberculosis is based on test and slaughter policy by application of cervical intradermal tuberculin test which is an approved OIE test for international trade **(OIE, 2009).**

In this study ,concerning to the examined samples collected from the Egyptian abattoirs ,the total isolates of mycobacteria recovered from offal samples from slaughtered tuberculin reactor cattle were 175 isolates(100%), all of them were from internal organs and lymph nodes and all of isolates were identified according to the morphological characters, growth rate, pigmentation and growth at different temperatures as M. bovis.

These results were higher than those obtained by **Labeeb (2011),** 62.9% and **Abed (2011),** 61.1%, and were less than those reported by **Soon (1981),** 92.1% and nearly similar to **Nasr et al. (2001),** 76.3%,

These results depend mainly on the actual disease status present in the tested herd, the experience of the investigators, as well as the technique used for decontamination of tissue specimens. Negative culture may be due to that tuberculous lesions are indistinguishable from lesions caused by other organisms that can be differentiated by histopathological examination, or due to death of M. bovis by macrophages **(O'Reilly, 1992)** or may be due to unsuccessful sampling from abattoir **(Araujo et al., 2005).**

The results of microscopical examination of different organs collected from tuberculin positive animals by Z.N stain revealed that 115 out of 175 examined samples showed acid fast bacilli and the lymph nodes had significantly higher number of positive results as 135/175(77%), followed by the spleen 20/175(11.2%) finally the liver and the kidney had the least number of positives for bovine tuberculosis (M. bovis) 10/175(5.7%). **(table 3)**

These results are in disagreement with those obtained by **Sa'idu et al., (2014)** who found that ZN microscopic staining 19/65(29.23%) of the lungs were positive for bovine tuberculosis as detecting of Acid Fast Bacilli while 6/28(21.43%) of the lymph nodes were AFB positive, followed by the heart, the intestines, the liver, and the spleen with the few AFB positive **(Cousins et al., 2004).**

As regards to the frequencies of isolation of mycobacteria from different samples, the obtained results proved that the highest rate was observed in lymph nodes samples followed by lung samples as shown in **(Table 2).**

PCR-based techniques are very important for the accurate differentiation of mycobacterial species and molecular epidemiological investigations of bovine tuberculosis **Michel et al., (2010),** that they is much faster than culture, reducing the time for diagnosis to 2 days and providing the ability to detect the presence of M. bovis in samples even when organisms have become nonviable for culture or low number of mycobacteria present in the sample, as well as they are more sensitive and specific. so, it was used on the collected samples and the obtained results revealed the detection of 175 positive samples out of 175 with percentage of detection reached 100% as shown in **(Table 2)**

Culture is considered to be the "gold standard", but this is a very slow and labor-intensive procedure and may become positive only several weeks after inoculation, especially for samples containing low numbers of mycobacteria.

It has been reported that PCR is 28 times more sensitive in the diagnosis of M. tuberculosis complex than traditional culture and direct microscopy **(Romero et al., 1999)** and the presence of the suspicious samples to be contaminated by high pathogenic bacteria.**(Riad,.et al., 2014)** The IS6110 gene - PCR has been reported to detect Mycobacterium tuberculosis complex in tissues samples by PCR assay and The results of Real Time PCR (RT-PCR applied on the extracted DNA of all isolates proved the ideal detection of mycobacterium spp. **(Taylor et al., 2007).**

Table (1) Results of bacteriological examination of L.NS. and offals of tuberculous animals by conventional methods at some Egyptian abattoirs

Post mortem inspection	Direct smear (Z.N stain)				Culture technique			
	Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%
175	115	65.7	56	34.3	137	78.3	38	21.7

Table (2) Results of PCR of examined organs and Offals samples at examined Abattoir

Source of Samples	No. of tested samples	PCR
		Positive
Aswan abattoir	100	100
Red sea abattoir	75	75
Total	175	175

(Table 3): Frequencies of isolation of mycobacteria from different samples at some abattoirs.

Type of samples	No. of tested samples	No. positive Samples
prescapular, prefemoral, axillary, poplitial, hepatic and renal lymph nodes	135	135
Kidney	10	10
Liver	10	10
Spleen	20	20
Total	175	175

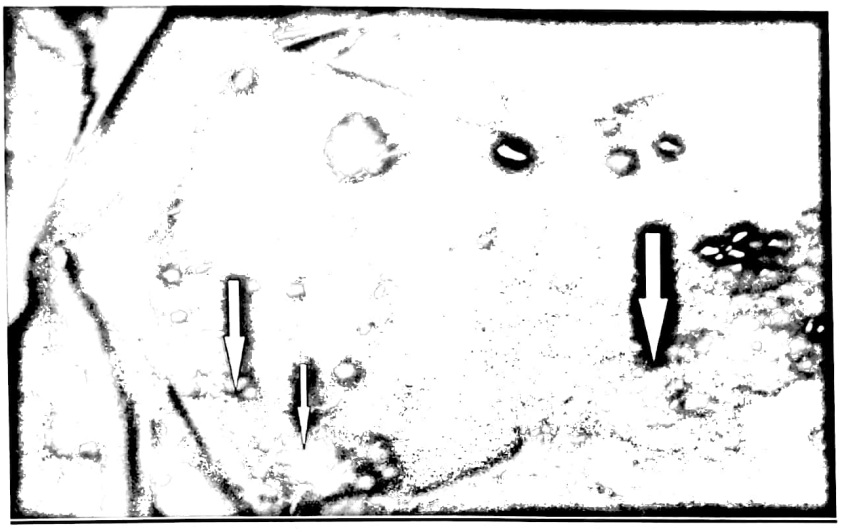

Photo (2): Liver showing suspected tuberculous lesions with calcification from slaughtered tuberculin reactor cattle.

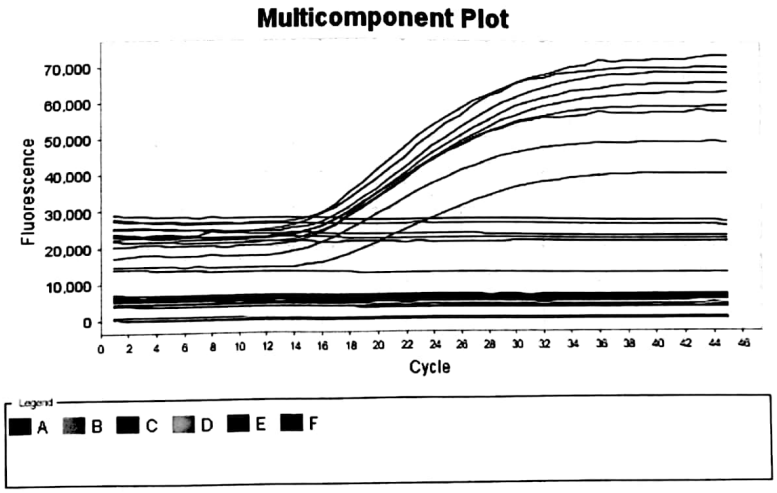

Figure (1): The amplification blot of suspected tissue samples Analysis for the amplification blot in its linear form: Eightsamples at cycle 14 and one control positive sample, six negative samples .The used reference dye is (FAM). The run is for 45 cycles.

Conclusion

We have to develop our national official laboratories / abattoir with diagnostic tests for mycobacterium tuberclosis.

Regular inspection to Slaughter houses and processing units to be insuring that applied the HACCP System.

The role of each stakeholders sharing in the food chain, must be studied and solve the problems in different levels and put plans for the development of meat industry

Change the awareness of food producers to increase the safety of product and awareness of consumers to avoid using low safety products or unknown products.

Training plan and seminars Extension for veterinary doctors and individuals who prepare and serve food to the public to

become responsible food handlers by using safe food handling methods and food preparation techniques and promote worker health and safety.

Improving our quarantine measures & laws which regulate the importing / exporting in the sector of products of animal origin as live animals & meat & meat industries which will have good influence in facilitating global trade.

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الملخص العربى

مدى تواجد ميكروب السل البقرى فى الاحشاء المستوردة والغدد الليمفاوية

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تعتبر المجازر مصدر قيم للمعلومات عن نسبة اصابة الحيوانات بالامراض التى منها الامراض المعدية والوبائية التى من الممكن ان تنتقل الى الانسان ومنها مرض السل البقرى الذى لايزال يمثل مشكلة صحية كبيرة فى الوقت الحالى.

تجري في كل وقت محاولات لتحسين سلامة الغذاء ، فيما يتعلق باستيراتيجية ذبح الابقار ويعتبر مرض السل البقرى من الأمراض شديدة الخطورة داخل المجازر ولذلك اجريت هذه الدراسة على عدد 175 عينة من الغدد الليمفاوية المختلفة والاحشاء الداخلية من الذبائح (الكبد – الطحال - الكلى) وذلك بتجميع العينات من الذبائح التى تم الكشف عليها ظاهريا بالمجزر بعد الذبح واشبهها بالاصابة بمرض السل البقرى وقد تم فحصها بالمعمل باستخدام الطرق التقليدية مثل (الزرع البكتيرى والفحص الميكروسكوبي) وايضا استخدام تقنية اختبار تفاعل البلمرة المتسلسل وقد اظهرت النتائج المعملية الاتى: عزل عدد 137 عترة يكتيرية تنتمى الى مجموعة ميكروبات السل من العينات وذلك بنسبة عزل (78.3 %) بينما كانت نسبة الكشف باستخدام تقنية اختبار البلمرة المتسلسل على نفس العينات بنسبة 100 % وذلك من العينات التى تم اعدامها بالمجازر من حيوانات ايجابية لمرض السل البقرى.

وقد اكدت هذة الدراسة دقة الاختبارات الحديثة وايضا كفاءة الاجراءات المجزرية فى الحكم على الذبائح داخل المجازر المصرية وايضا الاجابة على السؤال المقترح عن كيفية حماية صحة الشعوب النامية دون تعليق او منع الاستيراد.