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PREVALENCE OF MYCOBACTERIUM SPP. IN CATTLE MEAT AND OFFAL'S SLAUGHTERED IN AND OUT ABATTOIR

By

Shaltout, F.A.*, Riad, E.M and Asmaa Abou- Elhassan*****

*Faculty of Veterinary Medicine, food Control Dept. Banha Uni, **Animal Health Research Institute, Bacteriology Dept. ***General Organization for Veterinary services

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 one of the major health problem. A total of 360 meat samples, lymph nodes (prescapular L.N.and Prefemoral L.N. and Axilary L.N.) and internal organs (Livers, Kidney and Spleen) collected from carcasses after slaughtering of tuberculin positive animals were examined bacteriologically and the results of conventional culture techniques showed that 136 out of 180 examined samples were positive for isolation of mycobacteria (75.5%). and the rate of isolation at different abattoirs ranged from 73.3 % to 85 %. Concerning the collected samples out the target abattoir, 26 out of 180 examined samples were contaminated with *mycobacterium Spp.* (14.4. %). The results of real time PCR technique revealed that, all of tested tissue samples of 180 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. While the detection of contaminated meat with mycobacterium Spp. Proved the hygienic approach for slaughtering out the governmental abattoirs and the bad intended or non - intended selection of slaughtered animals from diseased one.

Key words:

Bovine tuberculosis, abattoirs, meat, diagnosis.

INTRODUCTION

Abattoirs can be source of valuable information of the incidence of animal diseases and conditions, some of which may be zoonotic. **(Phiri, 2006)** According to the World Health Organization report in 2013, tuberculosis still remains a major health problem with 9 million new cases and 1.5 million deaths annually worldwide **(WHO,2014).** Human tuberculosis caused by the bovine type **(Mycobacterium. bovis)** is scarce in developed countries, due to

control and regulation of meat products and the dairy industries; however, this is not the situation for the Third World. In less developed countries, Bovine tuberculosis has been identified in a wide variety of domesticated and non-domesticated animals **(WHO, 2009; and Zinsstag *et al.,* 2006)**. *Mycobacterium bovis* is the etiological agent of Tuberculosis in cows and rarely in humans. Both cows and humans can serve as reservoirs. Humans can be infected by the consumption of contaminated milk and eating of under cooked contaminated meat; this route of transmission can lead to the development of extrapulmonary TB. **(Todar's, 2013).** Human tuberculosis is a contagious-infectious disease mainly caused by *Mycobacterium tuberculosis*, which is an aerobic pathogenic bacterium that establishes its infection usually in the lungs. Like many other infectious diseases, TB presents epidemic cycles that might, although rarely, take centuries to end its course (**Rodrigo *et al.,* 2006)**. The eating of undercooked meat, and close contact with infected animals are the main sources of infection for humans. Bovine tuberculosis is a zoonotic disease caused by *Mycobacterium bovis* with potential public health and socio-economic significance as it can affect international trade in animals and animal product, it is a disease characterized by progressive development of specific granulomatous lesions or tubercles in lung tissue, lymph nodes or other organs. **(Ayele *et al.,* 2004).** In developed countries, eradication programs have reduced or eliminated tuberculosis in cattle, and human disease is now rare; however, reservoirs in wildlife can make complete eradication difficult, while control programs have eliminated or nearly eliminated this disease from domesticated animals in many countries. In Egypt prevalence of bovine tuberculosis in cattle was high in certain Governorates such as Alexandria (96%), Dakahlia and Behera was (9.6%) and (14.06%) during 1992, respectively. The aim of this study is directed to Screening the prevalence of *M. tuberculosis* in Egyptian abattoirs as well as to improve the diagnostic tools of *M. tuberculosis* in meat and offal's and to detect the prevalence of Mycobacterium tuberculosis in meat and offal's in some Egyptian governorates including Cairo, Giza and Kalubia, as well as its molecular diagnosis in the infected tissue using PCR.

MATERIAL AND METHODS

Samples:

A total of 360 A total of 360 meat samples, lymph nodes (prescapular L.N.and Prefemoral L.N. and Axilary L.N.) and internal organs (Liver, Kidney and Spleen) from slaughtered animals (in and out) of some governmental abattoirs (Giza, Cairo, Kaluobia).

(Table A): The types and numbers of the collected samples from different examined governmental abattoirs.

Gov.	Samples from cattle slaughtered in abattoir		Samples from cattle slaughtered outside abattoir		Total
	Type	No.	Type	No.	
Cairo	Meat (Prescapular L.N. & Prefemoral L.N. & Axilary L.N.)	30	Meat (Prescapular L.N. & Prefemoral L.N. & Axilary L.N.)	30	120
	Liver	10	Liver	10	
	Kidney	10	Kidney	10	
	spleen	10	spleen	10	
Gov.	Abattoir samples		Non- Abattoir samples		Total
	Type	No.	Type	No.	
Giza	Meat (Prescapular L.N. & Prefemoral L.N. & Axilary L.N.)	30	Meat (Prescapular L.N. & Prefemoral L.N. & Axilary L.N.)	30	120
	Liver	10	Liver	10	
	Kidney	10	Kidney	10	
	spleen	10	spleen	10	
Kaluobia	Meat (Prescapular L.N. & Prefemoral L.N. & Axilary L.N.)	30	Meat (Prescapular L.N. & Prefemoral L.N. & Axilary L.N.)	30	120
	Liver	10	Liver	10	
	Kidney	10	Kidney	10	
	spleen	10	spleen	10	
Total	180		180		360

Collection of samples:

Collection of lymph nodes and infected tissue samples:

A total of 360 A total of 360 meat samples, lymph nodes (prescapular L.N. and Prefemoral L.N. and Axilary L.N.) and internal organs (Liver, Kidney and Spleen) from slaughtered animals in and out of some governmental abattoirs at the period from September 2014 to June 2016. The samples were placed in sterile plastic bags and transferred directly to the laboratory in an ice box.

Preparation of samples:

Preparation of tissue samples and bacteriological isolation of mycobacteria; it was carried out according to Marks, 1972:

Organs and lymph nodes showing gross lesions were shopped into small pieces under aseptic conditions and 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 was then added and crushing was completed till the sample became a suspension. Then, 2 ml of 4% conc. H_2SO_4were added and incubated for 30 m, then diluted in 16 ml of sterile distilled water and centrifuged at 3000 rpm for 20 min. The supernatant was decanted into 5% phenol and the sediment was used for direct smear and inoculated onto 4 ml of Lowenstein Jensen media slant then aerobically 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 (Kubica, 1973).

Morphological characters:

Smears from expected colonies were made and 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 diagnosis of *Mycobacterium tuberculosis* complex:

The infected samples were examined by RT-PCR as follow.

Preparation of the samples for DNA extraction:

Each piece of infected tissue was homogenized in PBS (0.14M NaCl, 4mM KCl, 8mM Na2HPO4, 2mM KH2PO4, pH 6.5 buffers 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: Transfer lysate to purification column, centrifuge for 1min./ 8000 rpm, discard the collection tube then place column into new collection tube

3.Washing: Add 500 ul wash buffer 1 , Centifuge 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.

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'-CAGGGATCCACCATGTTCTTAGCGGGTTG-3'.

Reverse 5'-TGGCGAATTCTTACTGTGCCGGGGG -3'.

(Xiu-yun *et 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 Taq Man 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 qPCR 2x, 1 µl MTplexdtec-q PCR-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 Biosystems 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 large number of species, including humans it's also of great economic and sanitary importance in developing countries. **(Cobner, 2003)**.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)**. On the other hand, The intradermal tuberculin has been the widest used diagnostic technique although it has some sensitivity and specificity deficiencies and requires a second inspection of the animal for its interpretation beside the three traditional diagnositic techniques used for tuberculosis diagnosis in slaughtered animals (post mortem examinations, Ziehl Nelsen stain and bacteriological culture) **(Retmal and Abalos, 2004).**It allows detection of cattle that have been exposed to *M. bovis*. However, in herds where control of T.B is based on the identification and removal of reactors to this test, some animals in advanced stages of the disease and with open lesions don't show reactivity to tuberculin (anergic) and might remain in the herd, thus constituting a potential source of infection in susceptible cattle (**Diaz-Otero, *et al.,* 2003).** It is clear from (Table 1) that, the microscopical examination of 180 slaughtered tuberculin positive animals by direct smear stained by Ziehl-Nelseen stain revealed the detection of the acid fast bacilli in 114 examined animal samples with a percentage of (63.3%). The results of conventional culture techniques showed that, the bacteriological examination of tested samples internal organs (Livers, Kidney and Spleen) of tuberculin positive animals from each tested governorate revealed that 136 out of 180 examined samples were positive for isolation of *mycobacterium Spp.* (75.5%).as shown in (Table 1). Regarding to conventional culture of different organs (Livers, Kidney and Spleen), the rate of isolation at different abattoirs ranged from 73.3 % to 85 %.as shown in (Table 3). Concerning the collected samples outside the abattoirs the microscopical examination proved the detection of 16 out of 180 samples (8.8%) as shown in (Table 2).While the culture method can detect 19 out of 180 positive samples (10.5%) were contaminated with *mycobacterium Spp.* (Table 4). The result illustrated in (Table 1**)** showed that the examination of tuberculin reactor at different abattoirs as the results of microscopical examination of the 180 processed samples from the slaughtered tuberculin reactor cattle by Ziehl-Neelsen staining as in (Table 1**)**

showed the bacilli in only 114 (63.3%) samples. This result is higher than **(Abed, 2011)** 36.1%. Although microscopical examination of Acid Fast Bacilli is simple, inexpensive, and relatively quick procedure of detecting active tuberculosis, it does not identify the *Mycobacterium* species **(Vitale *et al.,* 1998)**, and need a bacterial load greater than 10^4 bacilli per ml uniformly distributed for reliable detection **(Tiwari *et al.,* 2007)**. Also in (Table 3) the total acid fast bacilli isolated from the samples of slaughtered tuberculin reactor cattle were 136 (75.3%) and all of them were identified according to the morphological characters, growth rate, pigmentation and growth at different temperatures as *M. bovis*. These results is higher than that obtained by **Labeeb (2011)**, as 62.9% and **Abed (2011)**, as 61.1%, and were less than that 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 and Cousins *et al.,* 2004),** or may be due to unsuccessful sampling from abattoir **(Araujo *et al.,* 2005)**. As regards to the results of PCR on the collected samples outside the governmental abattoirs, the results showed that 26 out of 180 tested samples where positive for *mycobacterium Spp.* Contamination (Table 5)**.**

Table(1): Results of bacteriological examination lymph nodes (prescapular L.N.and Prefemoral L.N. and Axilary L.N.) and internal organs (Livers,Kidney and Spleen)of tuberculous animals by conventional methods at some Egyptian abattoirs.

No. of tested samples	Direct smear (Z.N stain)				Culture technique			
	Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%
180	114	62.3	66	36.7	136	75.50	44	24.5

Table (2): Results of bacteriological examination lymph nodes (prescapular L.N., Prefemoral L.N.and Axilary L.N.)and internalorgans(LiversKidneyandSpleen) of tuberculous animals by conventional methods were collected out some Egyptian abattoirs.

No. of tested samples	Direct smear (Z.N stain)				Culture technique			
	Positive		Negative				Positive	
	No.	%		No.	%		No.	%
180	16	8.8	180	16	8.8	180	16	8.8

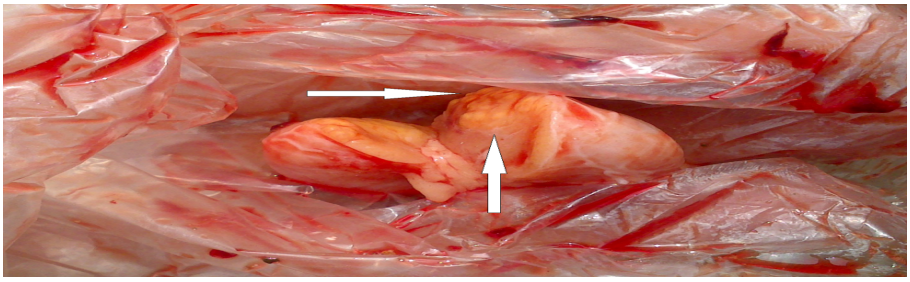

Photo (1): Bronchial L.N showing suspected tuberculous lesions with calcification.

Table (3): Results of cultural method of examined different lymph nodes (prescapular L.N., Prefemoral L.N. and Axilary L.N.) and internal organs (Livers, Kidney and Spleen) samples at different examined Governorate.

SOURCE OF SAMPLES	No. of tested samples	Culture technique	
		Positive	
		No.	%
Cairo	60	44	73.3
GIZA	60	41	68.3
KALUBIA	60	51	85
TOTAL	180	136	75.5

Table (4): Results of cultural method of examined different lymph nodes (prescapular L.N., Prefemoral L.N. and Axilary L.N.) and internal organs (Livers, Kidney and Spleen) samples were collected out some Egyptian abattoirs.

SOURCE OF SAMPLES	No. of tested samples	Culture technique	
		Positive	
		No.	%
Cairo	60	4	6.6
GIZA	60	9	15
KALUBIA	60	6	10
TOTAL	180	19	10.5

Results of real time PCR:

The results shown in (Table 5) revealed that by using of real time PCR technique, all tested tissue samples of tuberculin positive animals were positive with percentage of (100%), on the other hands PCR results of the collected samples out the governmental abattoirs showed that 26 out of 180 examined samples were contaminated with *mycobacterium Spp.* (14.4 %).

Table (5): The results of real time PCR assay of lymph nodes (prescapular L.N. , Prefemoral L.N. and Axilary L.N.) and internal organs (Livers , Kidney and Spleen) were collected from slaughtered animals (in , out) of some Governmental abattoirs.

Tissue samples	No. of tested samples	Real time-PCR	
		Positive No.	%
In abattoirs	180	180	100
out abattoirs	180	26	14.4

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

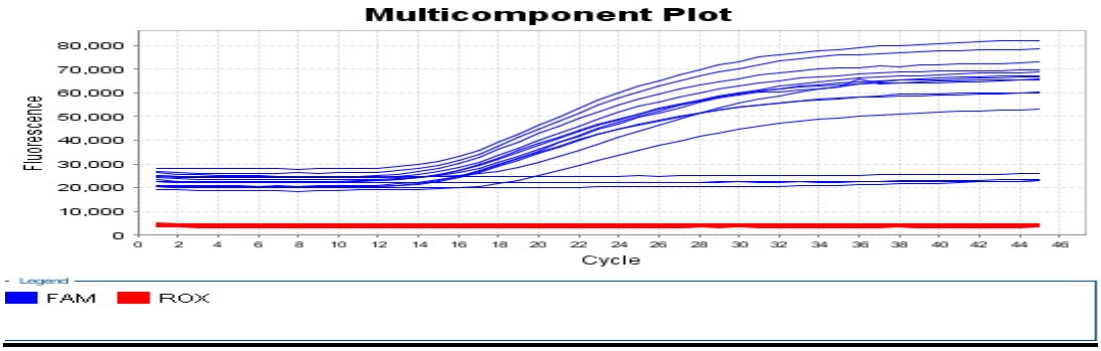

Fig. (1): Multicomponent curve of real time PCR result of mycobacterium bovis isolates.

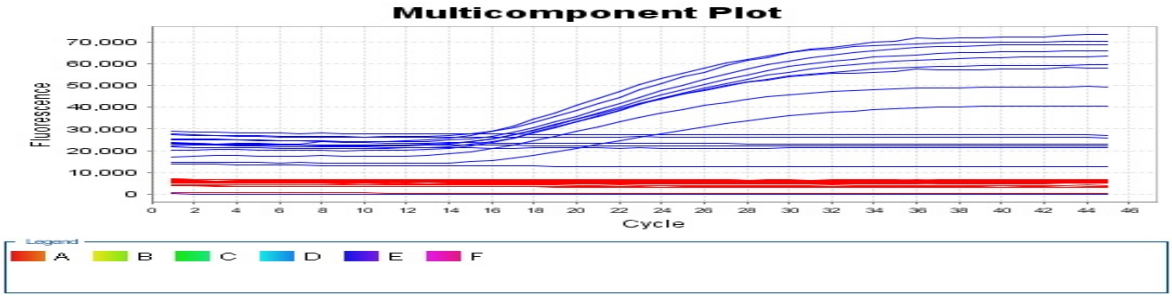

Fig. (2): The amplification blot of suspected tissue samples Analysis for the amplification blot in its linear form: Eightsamples at cycle 14 and one control positive sample, negative samples .The used reference dye is (FAM).The run is for 45 cycles.

The Comparison between results of microscopical examination, Culture technique and PCR assay proved that, the results shown in (Table 6) revealed that out of 180 tissue samples 114 were positive by direct smear with percentage (63.3%), while 136 were positive by Culture technique with percentage (75.3%), while all samples were positive by RT-PCR.

Table (6): Comparison between results of microscopical examination, Culture technique and PCR assay for samples were collected from some Egyptian abattoirs.

Types of samples	No. of tested samples	Direct smear (Z.N stain)		Culture technique		RT-PCR	
		Positive		Positive		Positive	
		No.	%	No.	%	No.	%
Tissue samples	180	114	63.3	136	75.3	180	100

Table (7): Comparison between results of microscopical examination, Culture technique and PCR assay for samples were collected out some Egyptian abattoirs.

Types of samples	No. of tested samples	Direct smear (Z.N stain)		Culture technique		RT-PCR	
		Positive		Positive		Positive	
		No.	%	No.	%	No.	%
Tissue samples	360	16	8.8	19	10.5	26	14.4

In this study the use of real time PCR revealed that all tested samples were positive for tuberculosis .which proved to be very important for the accurate differentiation of mycobacterial species and molecular epidemiological investigations of bovine tuberculosis **(Michel *et al.*, 2010),** that it 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 **(Shitaye *et al.*, 2006).(Reddington *et al.*, 2011)** or when there is an overgrowth by other mycobacteria or low number of mycobacteria present in the sample, as well as PCR techniques are more sensitive and specific. **(Riad *et al.*, 2014)**. While biochemical tests also could differentiate between distinct mycobacterial species, but they are very laborious, time consuming, and appear to be erroneous. 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 Taylor *et al.*, 2007)**. While the detection of contaminated meat with *mycobacterium Spp.* Proved the hygienic approach for slaughtering out the governmental abattoirs and the bad intended or non -intended selection of slaughtered animals from diseased one. While the detection of contaminated meat with *mycobacterium Spp.* Proved the hygienic approach for slaughtering out the governmental abattoirs and the bad intended or non -intended selection of slaughtered animals from diseased one.

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مدى تواجد ميكروب السل البقرى فى اللحوم والاحشاء من الذبائح داخل وخارج المجازر

فهيم عزيز الدين شلتوت –عماد مختار رياض – اسماء ابو الحسن

قسم مراقبة الاغذية - كلية الطب البيطرى جامعة بنها –معهد بحوث صحة الحيوان – الهيئة العامة للخدمات البيطرية

الملخص العربى

يعتبر مرض السل البقرى من الأمراض شديدة الخطورة داخل المجازر ولذلك اجريت هذه الدراسة على عدد 360 عينة لحوم وغدد ليمفاوية واحشاء داخلية من الذبائح داخل – خارج المجازر وذلك بتجميع العينات من الحيوانات الايجابية لاختبار التيوبركلين 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 136 عترة يكتيرية تنتمى الى مجموعة ميكروبات السل من العينات وذلك بنسبة عزل (75.5 %) بينما كانت نسبة الكشف باستخدام تقنية اختبار البلمرة المتسلسل على نفس العينات 100 % وذلك على العترات التى تم اعدامها والمأخوذة من العينات التى تم اعدامها بالمجازر من حيوانات ايجابية لمرض السل البقرى.
- عزل عدد 19 عترة بكتيرية تنتمى الى مجموعة ميكروبات السل من العينات بنسبة عزل (10.5 %) وذلك بالزرع البكتيرى بينما كانت نسبة الكشف باستخدام تقنية اختبار البلمرة المتسلسل على نفس العينات 14.4 %.

وقد اكدت هذة الدراسة دقة الاختبارات الحديثة وايضا كفاءة الاجراءات المجزرية فى الحكم على الذبائح داخل المجازر المصرية.