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Comparison of four different isolation and identification methods for *Mycobacterium bovis* from naturally infected cow milk with experimental infection of Guinea Pigs

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Mycobacterium bovis has a broad host range and is the principal agent responsible for tuberculosis in domestic and wild mammals, including cattle. Our study was designed to compare four different methods of isolation and identification of *M. bovis* from naturally infected-cow milk, and to evaluate the pathologic picture of experimentally infected guinea pigs with *M. bovis.* A total of 150 unique samples of raw cow milk were collected from 8 governmental dairy farms (100 cow milk samples) and 5 private dairy farms (50 cow milk samples) in Alexandria and El-Behera Governorates, Egypt during the period from July to October 2016. The results of microscopy (Ziehl-Neelsen stain), Modified Lowenstein-Jensen medium culture, Bio FM medium culture, and real-time PCR were positive in 6%, 8%, 4%, and 8% of the examined milk samples; respectively. Histopathologic study for experimentally infected guinea pigs revealed typical form of granuloma (yellowish appearance and calcified consistency) and microgranulomas, along with and necrotic and necrobiotic changes in liver, lungs, and kidneys. It could be concluded that PCR is at least as sensitive as microscopy, but had greater specificity because samples with atypical mycobacteria were not detected by PCR, it is also can overcome the drawbacks of other methods of diagnosis here.

Keywords: *Mycobacterium bovis*, Acid fast bacilli, Real-time polymerase chain reaction, Bact/Alert 3D System

INTRODUCTION

*Mycobacterium bovis*is a member of the "Mycobacterium tuberculosis complex" (MTBC), a group of genetically similar microorganisms that infect humans and animals. MTBC includes five named species (*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canettii*,and *M. microti*) and several microbes whose taxonomy is still debated (Brosch et al., 2002).

Unlike *M. tuberculosis*, which infects only humans, *M. bovis* has a broad host range and is the main agent responsible for tuberculosis in domestic and wild mammals, including cattle. Infection can potentially be spread to humans via contaminated milk, or directly by inhalation of aerosols from infected animals or carcasses (Cressey and Lake, 2009). Shedding of *M. bovis*

by an infected animal into oral and respiratory secretions and into feces can occur before a clinical diagnosis is made, and cross-contamination of milk batches from these animals may also occur (European Scientific Panel on Biological Hazards, 2003).

M. bovis affects all age groups and species of cattle. Infected cattle are the main source of infection, and inhalation is the major mode of transmission. Clinical findings in infected cattle are progressive emaciation, with signs depending on localization, e.g., respiratory distress, pharyngeal obstruction, reproductive disorder, or mastitis with the presence of tuberculous granulomas on necropsy (Radostits et al., 2010).

PCR testing is a fast and effective pathogen identification technique and has been widely used for detection of bovine mycobacteria, which grow slowly and are difficult to culture. Real-time PCR methods have been developed for the detection of bovine tuberculosis.

In Albania, Giuseppina and collagenous (Giuseppina et al., 2006) concluded that prevalence of MTBC in 50 raw cow milk samples is about 72%. However, (Moussa et al. 2005)examined 520 milk samples for MTBC using nested PCR analysis, thirty-three (6.35%) out of 520 tested positive for MTBC, where 23 (4.42%) of them were positive as confirmed by Ziehl-Neelsen (ZN) staining, and 14 (2.68%) of them were positive as confirmed by bacteriological examination. In addition, (Al-Saqur et al., 2009)examined 68 individual cow milk samples collected from AL-Fthelia-Baghdad for presence of MTBC, the positivity rate for a direct smear test was 4.4% (3 samples), while the positivity rate for the culture method was 10.2% (7 samples);results of the conventional methods were confirmed by PCR, and it yielded accurate positive results. (Shaimaa, 2009) detected *Mycobacteria* in 225 individual milk samples from tuberculin-positive reactors and 130 individual milk samples from tuberculin-negative reactors in El-Mansora, Egypt. The results indicated that 6 (2.7%) samples and 1 (0.8%) sample were positive for TB, respectively.

Another conventional method for identification of *M. bovis* is experimental infection of laboratory animals, infection of guinea pigs with *M. bovis* induced granulomatous tuberculous lesions in the lungs, liver, spleen, and kidneys. The lesions appeared in the 2nd weak post inoculation and gradually developed into extensive tuberculous granuloma with central caseation during the 4th and 6th weeks post inoculation. These lesions persisted and continued during the 8th week post inoculation (Al-Joboury, 2007).

The current study was initiated with two aims; the first aim is to compare the different methods of *M. bovis* isolation and identification from cow milk, and the second one is to investigate the experimental infection of guinea pigs with *M. bovis.*

MATERIALS AND METHODS

Samples

A total of 150 individual raw cow milk samples were collected randomly from 8 governmental dairy farms (100 individual cow milk samples) and 5 private dairy farms (50 individual cow milk samples) in Alexandria and El-Behera Governorates, Egypt. This study was conducted during the period from July to October 2016. Each sample (250 ml) was collected into a sterilized glass bottle and dispatched to the laboratory in an insulated ice box.

Chemicals

Carbol-Fuchsin solution according to Ziehl-Neelsen for microscopy (21820) was purchased from Sigma-Aldrich (Cairo, Egypt). Lowenstein Jensen (LJ) media (Cat. no. C21) was obtained from Hardy Diagnostics CO. Bio-FM medium and its supplement were ordered from (BIO-RAD, Marnesla Coquette, France, Ref 70,160–70,161). For Real-Time PCR, all reagents and enzymes were obtained from (Thermo Fisher Scientific, Waltham, MA USA 02451).Finally, all other materials and reagents used in this study were purchased from Sigma-Aldrich.

Detection of M. bovis

Microscopic detection of acid fast bacilli (AFB) by ZN staining.

Milk smears were prepared on clean and grease free slide, allowed to air dry and then heat fix. Smears were stained with ZN stain according to the method described by (Cruickshank, 1975).

Isolation of AFB on Modified Lowenstein-Jensen (L-J) media (glycerinated and pyruvated types).

After processing of the samples, the obtained precipitate was thoroughly mixed using a sterile Pasteur pipette before being evenly distributed into slants of glycerinated and pyruvated Modified L-J media in McCartney bottles (Ernst, 1990). The bottles were incubated in an upright position at 37°C and examined daily for 7 days and

periodically once a week for 6–8 weeks. The colonies of *M. bovis* were dysgonic: small, moist, and broken up easily on the pyruvated medium only.

Isolation of AFB on the Bio FM medium by means of a Bact/Alert 3D System (BioMérieux®, France)

Bio FM broth is a nonselective Middle Brook 7H9 medium, enriched with OADC (oleic acid, dextrose, and catalase), and optimized for growth of mycobacteria. Selectivity of the Bio FM medium was ensured by the addition of the Bio FM V.C.A. selective supplement (vancomycin, colistin, and amphotericin B) inhibiting the growth of the main gram-negative and gram-positive microorganisms as well as yeasts.

Procedures: one milliliter of a sample and 1 ml of a mix were added into a tube, and then centrifuged at 2500 rpm/10 min, and then the supernatant is discarded. Under aseptic conditions, 0.5 ml of a milk sample decontaminated with a syringe; and 1 ml of a substrate (an antibiotic supplement dissolved in 10 ml of a diluent) were added to the Automated TB bottles, the inoculated tubes were inserted into the Bact/Alert 3D System and assigned a code number (Savageet al. 1998).The result was obtained after 2–4 weeks.

Real-Time PCR (Genetic PCR Solutions - GPS)

a. The primers and probe were selected according to (Brosch et al. 1998).

F: CCC TTT CTC GTG TTT ATA CGT TTG A
R: GCC ATA TCG TCC GGA GCT T
P: CAC TCT GAG AGG TTG TCA
VIC-MGB fluorophores

(F, forward primer; R, reverse primer; P, probe).

b. Amplification conditions using the Hot Start-Mix qPCR 2X

Once all the reaction mixtures were ready, the tubes were pulsed-vortexed thoroughly, and the liquid was collected at the bottom by means of a pulse-spin in the centrifuge. Then, the tubes were placed in the block/rotor of the thermocycler programmed to run the cycling program shown in Table 1.

2.4. Experimental infection ofguinea pigs with *M. bovis*

Six healthy guinea pigs were infected by cultured L-J slants of *M. bovis*. The colonies were picked with a sterile small flat steel spatula and were weighed in a closed chemical weighing bottle. Next, 2.0 µg of the material was suspended in 10 ml of normal saline, and the suspension was kept in an amber-colored rubber-capped bottle at 4°C before use. After checking of suspension uniformity, 0.5 ml of suspension (representing 0.1 µg of bacilli) was injected intraperitoneally into each guinea pig, and then the injection was repeated after 2 weeks. Clinical signs and mortalities were reported. Dead animals were immediately necropsied and examined; specimens were collected from liver, lungs, and kidneys and fixed in neutral buffered formalin solution 10% for further histopathologic examinations (Varello et al. 2008). Fixed specimens were processed through the conventional paraffin embedding technique (Bancroft and Gamble, 2013). Several four-µm-thick sections were stained with haematoxylin and eosin (H.E) and histopathologic findings were evaluated microscopically. Representative micrographs were captured with a digital camera (Leica EC3, Leica, Germany) connected to a microscope (Leica DM500).

Statistical analysis:

All data frequencies were analyzed by Chi-square test using statistical analysis system *(SAS Institute Inc*, *2001)* to assess the significant differences between different groups at level of significant ($p<0.05$).

Table 1: Thermocycler program of Real-Time PCR.

45 cycles	Step	step	temperature
	Activation	5 min	95°C
	Denaturation	20-30 sec	95°C
	Hybridization, extension, data collection	45-60 sec	60°C

RESULTS

Incidence of *M. bovis* in raw cow milk samples examined microscopically by ZN stain.

As showing in Table 2and Fig. 1, the incidence of AFB among the individual raw cow milk samples stained with ZN stain and examined microscopically was6% (6 positive samples out of 100 samples collected from 8 governmental farms) and zero (zero positive samples out of 50 samples collected from 5 private farms).

Table 2: Incidence of Mycobacterium *bovis* among raw cow milk samples examined microscopically by Ziehl-Neelsen staining.

Milk samples	No. of Examined samples	Positive samples		Negative samples		
		No.	%	No.	%	
Governmental farms	100	6	6	94	94	3.12 NS (P>0.05)
Private farms	50	0	0	50	100	
Total	150	6	4	144	96	

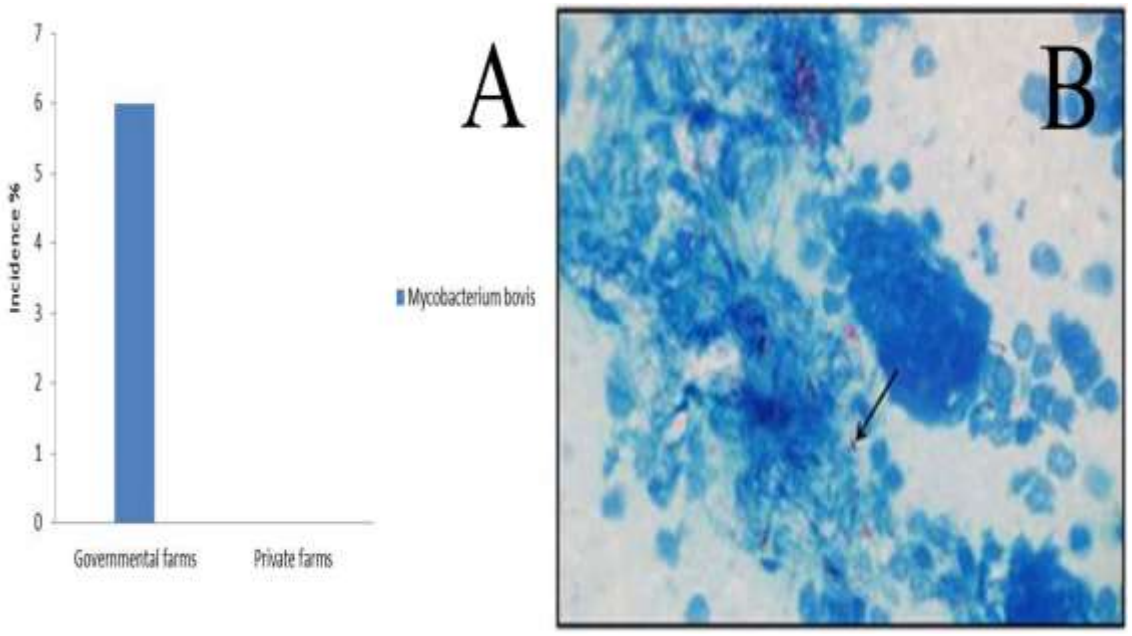

Figure 1: (A) Incidence of Mycobacterium *bovis* among raw cow milk samples examined microscopically by Ziehl-Neelsen staining.(B) Acid fast bacilli were stained with the Ziehl-Neelsen dye (Coccobacilli of M. *bovis*). They stained red with a blue background and were examined microscopically by means of an oil immersion lens (100).

Incidence of *Mycobacterium bovis* among raw cow milk samples (isolation using L-J media).
As showing in Table 3 and Fig.2, the incidence of *M. bovis* among the examined raw cow milk samples obtained from 8 governmental and 5 private farms by the conventional culture method (L-J medium, pyruvated type, that is specific for *M. bovis*) was 8% and zero, respectively, while the L-J medium, glycerinated type (that is specific for *M. tuberculosis*) showed no growth for both sources of milk.

Incidence of *Mycobacterium bovis* among raw cow milk samples as assessed by the Bact-alert device.
By conducting fast microbial testing, B act/Alert 3D delivers resulted in almost half the time required by conventional culture testing methods. The state-of-the-art detection system provides objective endpoints that are not affected by turbid samples. Sampling is nondestructive. As illustrated in Table 4 and Fig. 3, the Incidence of AFB among the raw cow milk samples from both governmental and private farms was 4% and zero, respectively.

Table 3: Incidence of Mycobacterium *bovis* among raw cow milk samples (isolation using Lowenstein-Jensen media).

Milk samples	No. of examined samples	Lowenstein-Jensen media			
		Pyruvated		Glycerinated	
		No. of positive samples	%	No. of positive samples	%
Governmental farms	100	8	8	0	0
Private farms	50	0	0	0	0
		4.22* (P<0.05)		NS (P>0.05)	
Total	150	8	5.33	0	0

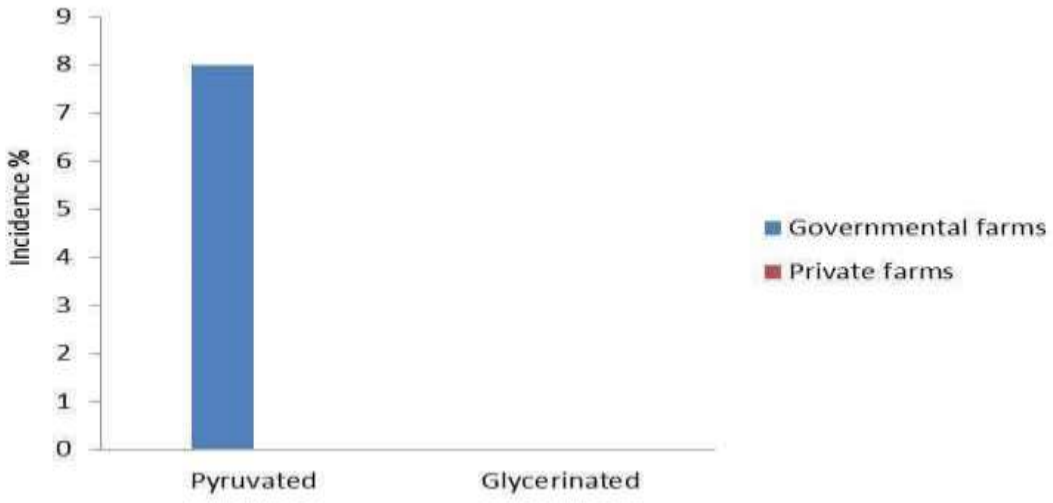

Figure 2: Incidence of Mycobacterium *bovis* among raw cow milk samples (isolation using Lowenstein-Jensen media).

Table 4: Incidence of Mycobacterium *bovis* among raw cow milk samples as assessed by the Bact-alert device.

Milk samples	No. of examined samples	Positive samples		Negative samples		
		No.	%	No.	%	
Governmental farms	**100**	**4**	**4**	**96**	**96**	**2.05NS (P>0.05)**
Private farms	**50**	**0**	**0**	**50**	**100**	

Figure 3: (A) Incidence of Mycobacterium *bovis* among raw cow milk samples as assessed by the Bact-alert device. (B) Mycobacterium *bovis*isolated on a Lowenstein-Jensen medium (pyruvated type) showing dysgonic, small, moist, colonies that break up easily (asterisk). (C) Acid fast bacilli isolated on the Bio FM medium by means of a Bact-alert device showing positive yellow sensor (black asterisk) and negative gray sensor (white asterisk).

Incidence of *M. bovis* among raw cow milk samples according to real-time PCR.

As showing in Table 5 and Fig. 4, the incidence of *M. bovis* among raw cow milk samples obtained from governmental and private farms is 8% and zero, respectively, according to real-time PCR.

Comparison among different methods of *M. bovis*detection.

Comparison between the results of microscopic, culture-based, and real-time PCR methods for detection of *M. bovis* in the examined raw cow milk samples obtained from governmental farms is shown in Table 6 and Fig.5. The incidence according to the microscopic, Modified L-J medium culture, Bio FM medium culture, and real-time PCR analyses was 6%, 8%, 4%, and 8%, respectively, among the examined individual raw cow milk samples obtained from 8 governmental dairy farms. (These percentages were reported on only one governmental dairy farm).

Findings of experimental infection of guinea pigswith M. *bovis*.

Postmortem findings

Following infection, gradual loss of weight was observed every week, and death occurred after 30–35 days. Postmortem examination revealed presence of tuberculous granuloma in the liver, spleen, lungs, thoracic cavity, small intestine, and kidneys; and the presence of AFB was examined by ZN staining (Fig. 6).

Histopathologic findings

Histopathologic examination to liver tissues revealed presence of micro granulomas that composed of necrotic center (Fig. 7a) contained basophilic structure less deposition of calcium salts (Fig. 7b), necrotic center was surrounded by mononuclear cells (predominantly epithelioid, and plasma cells and lymphocytes); the bile ductless showed proliferation of fibrous connective tissue (CT) around them and were infiltrated with mononuclear cells (Fig. 7c). The portal triad had a dilated hepatic artery (Fig. 7d) with fatty changes in the adjacent hepatocytes (Fig. 7e).

Examination of lung tissues showed formation of

multiple micro granuloma composed of a central necrotic zone surrounded by leukocytes (principally histiocytes and lymphocytes) and enclosed within thin fibrous CT capsule (Fig. 8a,b). The hemosiderin pigment (golden yellow) either appeared in pulmonary parenchyma or was engulfed by histiocytes (Fig. 8c). Pulmonary blood vessels showed edema around them, the bronchiolar lumen revealed aggregates of mononuclear cells and pulmonary emphysema (Fig.8d). Additionally, kidneys tissues showed necrobiotic changes (degeneration and necrosis) of the tubular epithelium, edema of interstitial tissue, and epithelial casts in the lumen of the renal tubules (Fig.8a,b).

Table 5: Incidence of M. *bovis* among raw cow milk samples according to real-time PCR.

Source of milk samples	No. of examined samples	Positive samples		Negative samples		
		No.	%	No.	%	
Governmental farms	100	8	8	92	92	4.22* (P<0.05)
Private farms	50	0	0	50	100	
Total	150	8	5.33	142	94.66	

Table 6 A: comparison among different methods of Mycobacterium bovis detection. This incidence of M. *bovis* as evaluated by different techniques was reported only for one governmental dairy farm.

Source of milk samples	No. of samples	Microscopic examination		Cultural exam.				Real-time PCR	
				L-J media (Pyruvated)		Bact/Alert 3D system			
		positive samples	%	Positive samples	%	Positive samples	%	Positive samples	%
Governmental farms	100	6	6	8	8	4	4	8	8
Private farms	50	0	0	0	0	0	0	0	0
		3.12 NS (P>0.05)		4.22* (P<0.05)		2.05NS (P>0.05)		4.22* (P<0.05)	
Total	150	6	4	8	5.33	4	2.67	8	5.33

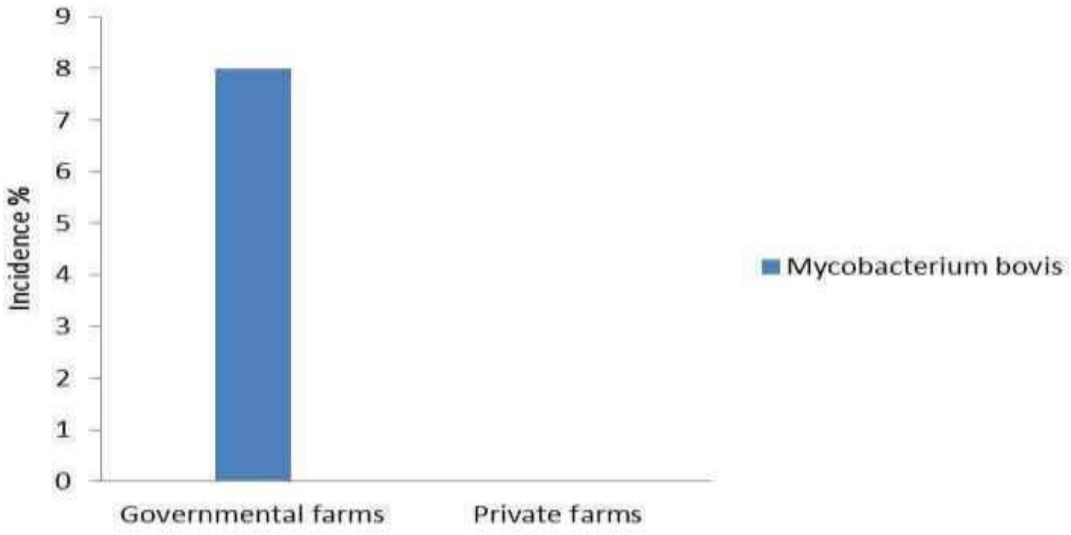

Figure 4: Incidence of M. *bovis* among raw cow milk samples according to real-time PCR.

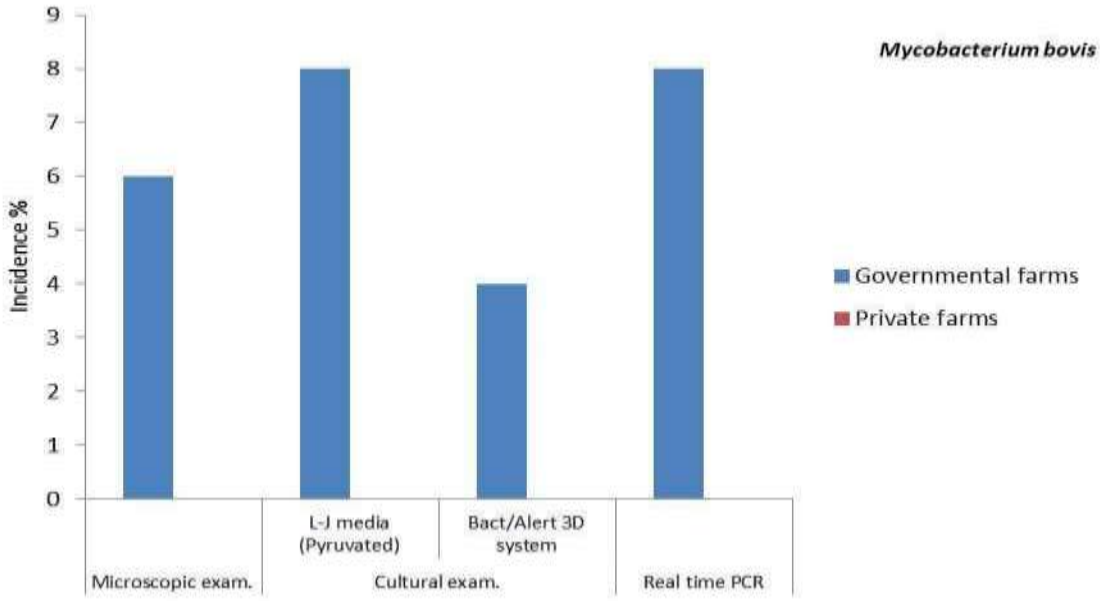

Figure 5: A comparisons among different methods of Mycobacterium *bovis* detection.

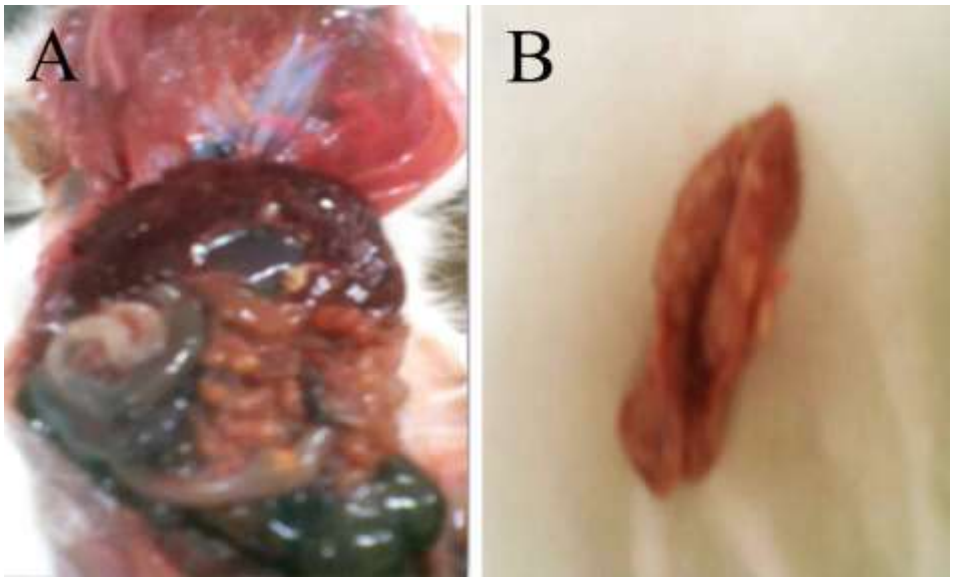

Figure 6a: A lesion of bovine tuberculosis showing generalized granulomatous reactions of TB in various organs (liver, thoracic cavity, and small intestine) of a guinea pig. Figure 6b; tuberculous granuloma in the duodenum

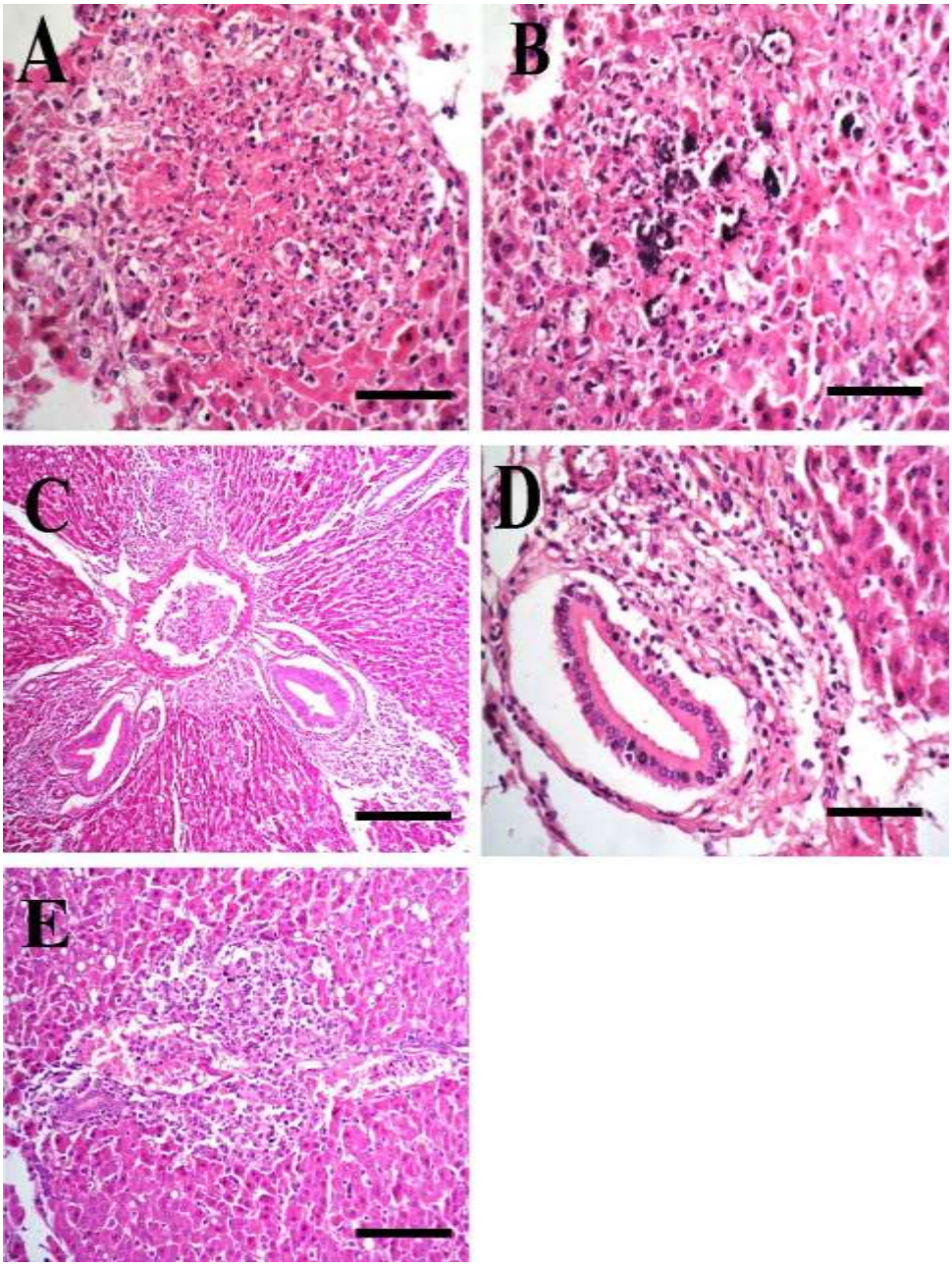

Figure 7: Liver of guinea pig infected with Mycobacterium *bovis* and stained with H.E revealing: (A) microgranulomas that composed of a focal necrotic zone surrounded by mononuclear cells, principally epithelioid and plasma cells and lymphocytes. (B) The central necrotic zone of micro granulomas showed basophilic structureless deposition of calcium salts.(C) Fibrous connective tissue proliferation around the bile ductules as well as mononuclear cells was noticed. (D) Dilatedbile ductules and (E) portal leukocyte cell aggregates were noticed, together with fatty change in the adjacent hepatocytes (*bar*= 50 µm for A, B, D- *bar*= 200 µm for C,E)

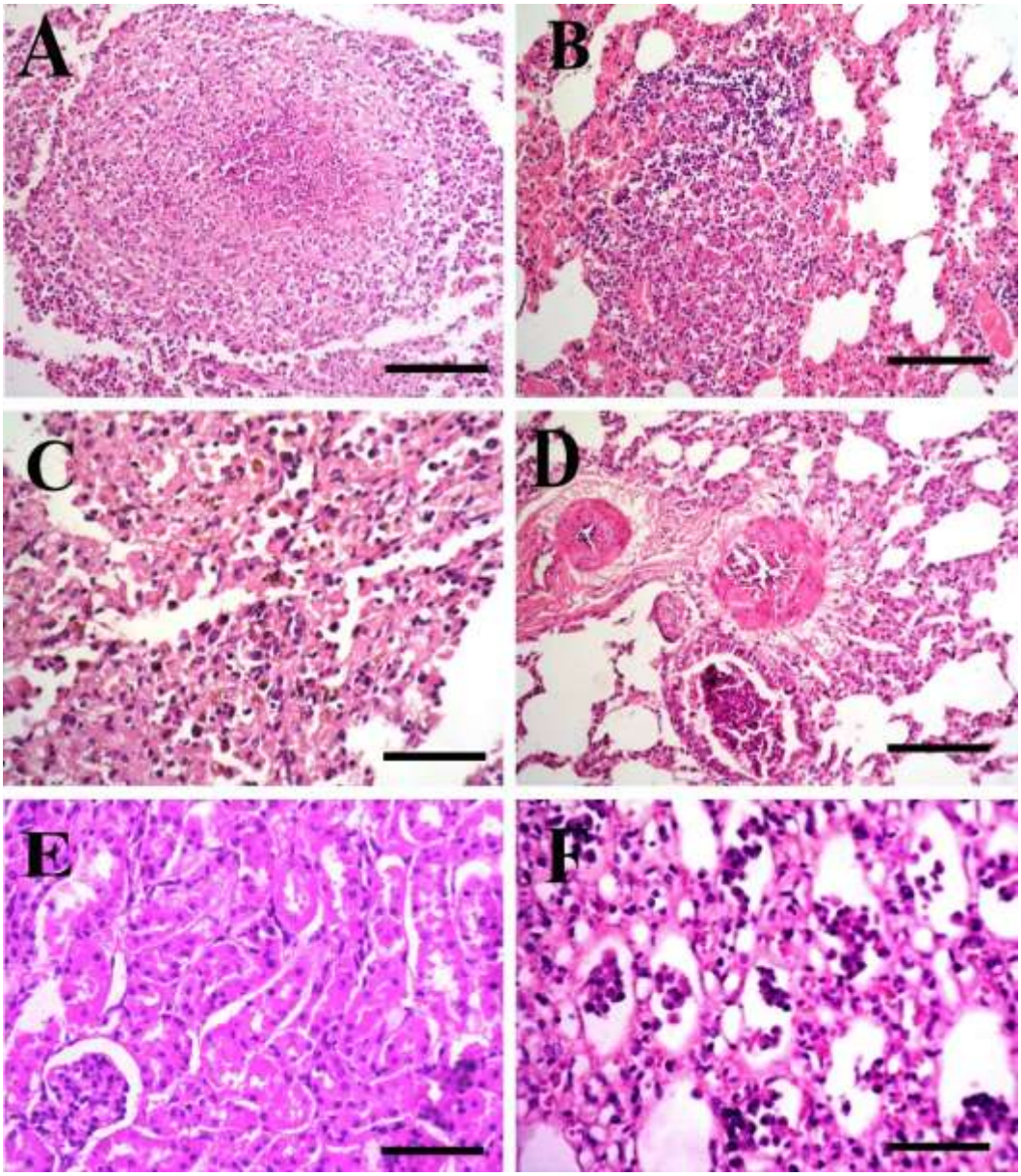

Figure 8;Lungs and kidneys of guinea pig infected with Mycobacterium *bovis* and stained with H.E revealing: (A, B) microgranulomas composed of central necrotic zone, and surrounded by leukocytes, principally histiocytes and lymphocytes, and thin fibrous connective tissue capsule. (C) Lung tissue showing hemosiderin pigment (golden yellow) that either appeared in pulmonary parenchyma or was engulfed by histiocytes. (D) Perivascular edema and pulmonary emphysema were evident.(E) Kidney of a guinea pig infected with M. *bovis* showing necrobiotic changes of the tubular epithelium and (F) epithelial casts in the lumen of renal tubules (*bar*= 50 µm for A,C,F- *bar*= 200 µm for B,D,E) .

DISCUSSION

Tuberculosis is a worldwide epidemic disease that resulting in significant morbidity and deaths of 2 million people per year (Raviglione, 2003) This disease is caused by members of the MTBC, which is consists of closely related species and subspecies that include *M. tuberculosis*, *M. bovis* (the causative agent of bovine tuberculosis), and *M. bovis* BCG (the live attenuated tuberculosis vaccine strain) (Ernst et al., 2007).

MTBC is diagnosed by bacteriological and histopathological techniques. Rapid nucleic-acid-based methodologies, such as PCR may also be used as a confirmatory method. Traditional mycobacterial culture remains the gold standard of routine confirmation of diagnosis (Office International Des Epizooties, 2009).

In the current study, the incidence of AFB among the individual raw cow milk samples as confirmed microscopically by ZN staining was 6% and zero in governmental and private farms, respectively. These results are lower than those reported by (Mehran et al., 1959). Nearly similar results were obtained by (Sami, 1980). However, the incidence of *M. bovis* among the examined raw cow milk samples by the conventional culture method (L-J medium, pyruvated type, that is specific for *M. bovis*) was 8% and zero in governmental and private farms, respectively, while the L-J medium, glycerinated type (that is specific for *M. tuberculosis*) showed no growth for both sources of milk. In the previous studies, higher incidence of infection (13.3%) was detected by (Ameni et al.,2003)in selected dairy farms in Ethiopia, whereas in Brazil, Clarice Fujimura (Leite et al. 2003) isolated mycobacteria from 23 of 128 (18%) individual cow milk samples. Also, (Hamid et al., 2003) found *M. bovis* in 25% of milk samples from tuberculin-positive cows. On the other hand, lower incidence was reported by (Olivera et al.1983) who detected positive presence (4.35%) of mycobacteria in 23 bovine milk samples.

In this study, by conducting fast microbial testing, Bact/Alert 3D (BioMérieux®) delivers results in almost half the time required by conventional culture testing methods. The state-of-the-art detection system provides objective endpoints that are not affected by turbid samples. Sampling is nondestructive. Because it's too easy to use, Bact/Alert 3D saves time, facilitates cross-training, and manages contamination. Bact/Alert Culture Media offer the ability to detect aerobic, anaerobic, and facultative anaerobic microorganisms. With the Bio FM medium (Bact-alert device), the Incidence of AFB among the raw cow milk samples from both governmental and private farms was 4% and zero, respectively.

PCR is the most sensitive and accurate test for detection of MTBC. PCR sensitivity was found to be 2–20 times better than that of bacteriological analysis in experimentally inoculated milk samples. Besides, culture-based protocols are known to be slow: taking several weeks to yield a diagnostic result. In contrast, molecular approaches based on PCR offer a more rapid interpretation. Several PCR-based approaches have been described and may be useful as an adjunct to the culture method (Perez et al., 2002, Zumarraga et al., 2005, Halse et al., 2011).Our presented results revealed that the incidence of *M. bovis* among raw cow milk samples obtained from governmental and private farms is 8% and zero, respectively, according to real-time PCR.

Comparing of the incidence of *M. bovis* in the examined raw cow milk samples (obtained from one governmental dairy farm)was 6%, 8%, 4%, and 8% as recorded by the microscopic, Modified L-J medium culture, Bio FM medium culture, and real-time PCR analyses; respectively. This results showed that detection of AFB by ZN staining (obtained prevalence rate 6%) was less effective than detection by culturing on the pyruvated L-J medium (obtained incidence rate 8%), and more effective than the detection by manual and automated culturing by means of the Bio FM and Bact/Alert 3D System (obtained incidence rate 4%). From these results, it was clear that higher and/or lower results on AFB obtained by microscopic examination compared with both types of culture analysis may be attributed to the prolonged specimen decontamination and incubation in cultures; these factors can also explain smear positive and culture negative results (Murrary et al., 1980). ZN staining is not specific for *Mycobacteria* spp., and may detect other species of AFB (Kubica, 1973).

The results of culture examination of *M. bovis* indicate that the L-J medium culturing (obtained incidence rate 8%) was more effective than automated culturing by means of the Bio FM Bact/Alert 3D System (4%) (Spahr and Url,1994) and this situation explains the higher and lower results detected by both types of culture examination because the L-J medium requires a large milk sample (100 ml); this requirement resulted in a large amount of the precipitate (a sufficient number of tuberculous bacilli can be detected). In contrast, in case of automated culturing by means of the Bio FM Bact/Alert 3D

System, a small milk sample is used (1 ml), and the resulting precipitate is small (a small number of tuberculous bacilli will be detected).

The results of real-time PCR analysis for detection of *M. bovis* in raw cow milk samples yielded the incidence rate of 8%: higher than that obtained by automated culturing on the Bio FM Bact/Alert 3D System or by microscopic examination (4% and 6%, respectively), and equal to the rate obtained by L-J medium culturing.

PCR is a sensitive and fast diagnostic tool that can be used to detect a pathogen in clinical samples within 48 hours, but the presence of inhibitors in samples can interfere with PCR performance (Haddad et al. 2004, Singh et al. 2004, Brasil, Ministerio da Agricultura, Pecuaria e Abastecimento, 2005). The sensitivity and accuracy of PCR are greater than those of culture analysis (Sakamoto, 1997), and therefore PCR is more confirmatory for isolates. Genetic elements specific to virulence of the target species constitute attractive probe targets. The presence of a virulence factor can help to determine the disease potential of various samples (Lang et al., 1994). The genetic elements responsible for the virulence of mycobacteria are unclear. Many virulence genes of various levels of complexity are probably present in MTBC.

In this study, infected guinea pigs developed progressive tuberculosis and died 35 to 40 days after infection with *M. bovis*, thus showing almost uniform susceptibility to infection. The present findings are similar to those of (Mangalik et al., 1954)who reported that all animals died by the 35th day after a single intramuscular inoculation of 1 mg of MTBC .Postmortem examination of different organs of guinea pigs showed generalized form of tuberculosis; in which all organs (the liver, lungs, spleen, kidneys, small intestine, abdominal muscles, and diaphragm) showed multiple necrotic areas, congestion, and a typical form of granuloma which had a yellowish appearance and was caseous, caseo-calcareous, or calcified in consistency. Extra-pulmonary lesions were prevalent in this experiment.

Histopathological examination of liver, lungs, and kidneys showed multiple micro granulomas, necrotic, and necrobiotic changes. These results were similar to those obtained by (Gillespie and Timoney, 1981, Grange, 1995, Quinn et al., 1999).Granulomatous lesions can be caused by other infectious agents such as fungi, *Staphylococcus*, Actinomyces, and Actinobacillus spp.; by foreign bodies; and by the presence of calcified pus, as is commonly the case for Actinomyces(Cousins et al.2004). Therefore, we confirmed our findings by subjecting granulomatous lesions to ZN staining.

CONCLUSION

PCR was at least as sensitive as microscopy, but had greater specificity because samples with atypical mycobacteria were not detected by PCR; the latter method overcame all the drawbacks of other methods of diagnosis here.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

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AUTHOR CONTRIBUTIONS

The authors declare that they have no conflict of interest.

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