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Highlighting on *Mycobacterium Bovis* in cattle, focusing on its antimicrobial resistance

El-Gedawy, A .A.*, Abeer E. Abdelghafar **,Yousry A. El-Shazly*, Mohamed A. Elkady**, Mahmoud A. Abdelrahman** and Marwa Shalaby****

*Bacteriology Dept., Animal Health Research Institute, Dokki. **Bacteriology Dep., AHRI, Mansoura.
***Veterinary Hospital, Faculty of Vet. Med., Zagazig university.

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ABSTRACT

The current state of multidrug resistance among *Mycobacterium bovis (M.bovis)* isolates originating from cattle in Egypt underscores the need for control measures based on accurate diagnostic approaches. Conducting antibiogram surveillance of *M.bovis* using advanced molecular techniques can greatly contribute to the prevention of transmission of multi-drug-resistant (MDR) strains to humans. In this study, a total of 153 tuberculous lesion samples were collected from slaughtered cows in the abattoir and subjected to bacteriological examination. Acid-fast bacilli were detected in 54.2% of samples using ZN staining, while M.bovis was isolated from 65.4% of samples through culture. Out of the 65 M.bovis isolates confirmed by PCR, 25 showed resistance to at least one anti-tuberculous drug. The resistance rates to EMB, STR, INH, and RIF were 3.1%, 20%, 30.8%, and 32.3% respectively. MDR was detected in 21.5% of isolates. DNA sequencing of six rifampicin-resistant isolates revealed three specific mutations (H526Y, S531L, and D516V). These findings highlight the importance of investigating ante-mortem *M.bovis* infection in cattle to minimize public health risks associated with bovine tuberculosis.

INTRODUCTION

Bovine tuberculosis (BTB) is a chronic disease in cattle caused by Mycobacterium bovis *(M.bovis),* a member of the Mycobacterium tuberculosis complex (MTBC). It has significant economic and public health implications due to its impact on animal production and the potential for human infection **(Mohamed 2020)**. In humans, tuberculosis (TB) is a major global health concern **(WHO 2017)**. M.bovis accounts for approximately 10-15% of TB cases in developing countries. The emergence and spread of multidrug-resistant TB (MDR-TB), particularly resistance to rifampicin (RR), is a serious threat to TB control efforts.

Isoniazid (INH) resistance is caused by different mutations affecting different genes **(Vilchèze et al. 2014)**, while on molecular basis rifampicin (RIF) resistance is less difficult, as absolutely all resistant strains exhibit rpoB gene mutations **(Telenti et al. 1993)**. Moreover, many countries use rifampicin resistance detec-

Corresponding author: El-Gedawy, A .A, Bacteriology Dept., Animal Health Research Institute, Dokki, Egypt.
E-mail: Dr.attia31@yahoo.com
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tion by molecular techniques as a potential marker of MDR-TB, because more than 90% of cases resistant to rifampicin exhibit resistance to isoniazid **(Mboowa et al. 2014)**.

This study aimed to isolate MTBC from animal tissue samples obtained after postmortem examination, confirm *M. bovis* using PCR targeting Mpb70, assess the drug sensitivity of isolates, and investigate rifampicin resistance mutations through DNA sequencing.

MATERIALS AND METHODS

1.1 Collection of Samples:

A total of 153 tissue samples, exhibiting tuberculous lesions, were gathered from slaughtered cows that tested positive for tuberculin in the abattoir after postmortem examination, following the guidelines set forth by the Egyptian authorities for cattle inspection.

2.1 solation and Identification:

The tissue samples were processed and inoculated into modified LJ medium (Oxoid, England) as instructed by the manufacturer **(Marks 1972)**. Additionally, direct smears of the processed samples were stained with Ziehl-Neelsen (ZN) stain according to the manufacturer's instructions **(Kubica 1973)**

3.1Confirmation of *M.bovis* by Conventional PCR Targeting Mpb70:

DNA extraction from 65 selected isolates was conducted using the QIAamp® DNA Mini Kit (Qiagen, Germany) following the manufacturer's guidelines. These isolates were then subjected to PCR using primers designed to target the mpb70 gene: forward (5'-ACCCTCAACAGCGGTCAGTAC-3') and reverse (5'-TTACGCCGGAGGCATTAGCAC-3'). This specific PCR amplifies a 314 bp product that is specific to *M.bovis*, as described by **Zhang et al. (2016)**.

4.1 Antibiogram by the Gold Standard Agar Proportion Method in Middlebrook 7H11 Medium:

The susceptibility of the 65 M.bovis isolates, confirmed by the presence of Mpb70, to certain anti-mycobacterial drugs was determined using the gold standard agar proportion method in Middlebrook 7H11 medium. This method follows the approved standard (M24A) provided by the Clinical and Laboratory Standards Institute **(CLSI 2011)** for the susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes.

5.1 PCR and Sequencing for rpoB gene of Some Drug-Resistant *M.bovis* Isolates:

DNA from six selected isolates showing resistance to rifampicin (Code N0:M-LN-2, M-LN-9, M-LN-12, S-LN-5, S-LN-7, and Q-LN-8) was amplified using PCR with primers targeting the rpoB gene: forward (5'-GGAGCGGATGACCACCC-3') and reverse (5'-GCGGTACGGCGTTTCGATGAAC-3'). This PCR amplifies a 350 bp product specific for rifampicin-resistant isolates, as described by **Siddiqi et al. (2002)**. The PCR products were purified with the QIAquick PCR Product extraction kit (Qiagen Inc. Valencia CA) and then sequenced in both directions using the Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, FC, CA). Purification of the sequencing reaction was performed using Centrisep (spin column), and the sequencing reaction was carried out on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). Sequence comparisons were conducted following the method

RESULTS

Table 1. showed the bacteriological examination of 153 processed samples from El-Menufia, El-Sharkia, and El-Qaliobia Governorates

Governorate	No. of tested samples	AFB by Direct smear (Z.N)		Positive culture	
		NO.	%	NO.	%
El-Menufia	69	38	55.1	44	63.8
El-Sharkia	47	25	53.2	31	66
El-Qaliobia	37	20	54.1	25	67.6
Total	153	83	54.2	100	65.4

described by **Thompson et al. (1994)**. Phylogenetic analysis utilizing neighbor joining, maximum likelihood, and maximum parsimony methods in MEGA6 was performed, as outlined by **Tamura et al. (2013)**

Results of conventional PCR targeting Mpb70

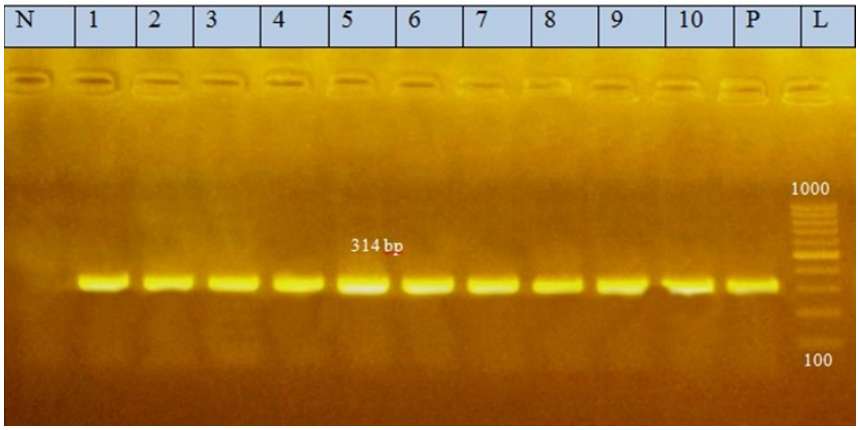

Fig (1): Agarose gel electrophoresis for PCR amplified products of *M.bovis* isolates
Lane (L): DNA ladder. Lane (P.): positive control. Lane (N.): Negative control.
Lane (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10): Positive *M.bovis* isolates with specific amplicon size 314bp.

The antibiogram results of 65 *M.bovis* isolates confirmed by Mpb70 targeting PCR showed that 61.5% of the isolates were pan-susceptible to the tested anti-TB drugs. However, 38.5% of the isolates exhibited resistance to at least one of the drugs. The resistance percentages for ethambutol, streptomycin, isoniazid, and rifampicin were 3.1%, 20%, 30.8%, and 32.3%, respectively. Additionally, 21.5% of the isolates were identified as MDR-*M.bovis*

Table 2. Result of sensitivity of *M.bovis* isolates against antituberculous drugs

No. of tested isolates	Tested antimicrobial agent								No. of pan-sensitive isolates	
	Isoniazid sensitivity		Rifampicin sensitivity		Streptomycin sensitivity		Ethambutol sensitivity			
	No.	%	No.	%	No.	%	No.	%	No.	%
65	45	69.2	44	67.7	52	80	63	96.9	40	61.5

Results of PCR for some drug resistant isolates:

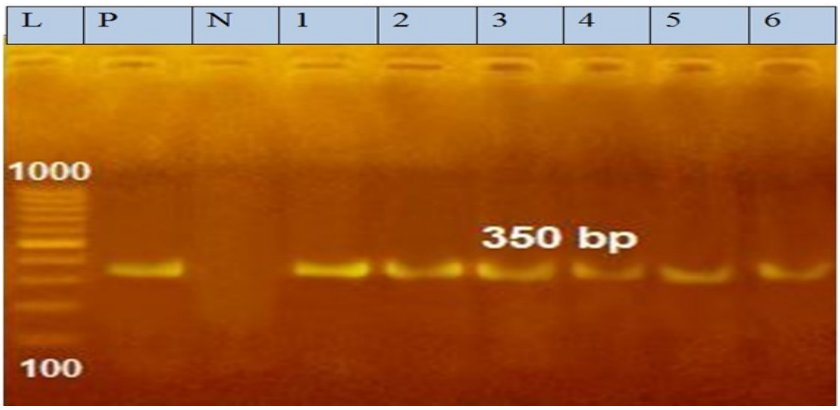

Fig (2): Agarose gel electrophoresis for PCR amplified products rpoB gene of of the resistant *M.bovis* isolates.
Lane (L): DNA ladder. Lane (P.): positive control. Lane (N.): Negative control.
Lane (1, 2, 3, 4, 5 and 6): positive isolates for *rpo*B resistance gene of rifampicin with specific amplicon size 350 bp.

Results of sequencing PCR products from six *M.bovis* isolates, indicating rifampicin resistance. Among these isolates, three nucleotide substitution mutations were detected: D516V in 1 out of 6 isolates, H526Y in 4 out of 6 isolates, and S531L in 1 out of 6 isolates

Table 3. Amino acid changes in resistant isolates:

Isolate ID	Resistance pattern	gene	Affected codon	Amino-acid change
M-LN-2	R	rpoB	531	S-----L
M-LN-9	R,I		526	H-----Y
M-LN-12	R,I		526	H-----Y
S-LN-5	R,I,S,E		526	H-----Y
S-LN-7	R,I,S		526	H-----Y
Q-LN-8	R		516	D-----V

R: rifampicin,I: Isoniazid, S: streptomycin, E: ethambutol

DISCUSSION

Bovine tuberculosis remains a significant global threat to cattle, making it crucial to control and prevent outbreaks of MDR-M.bovis and its transmission to humans **(Franco et al., 2017)**. Table 1 demonstrates that the results of directsmear in this study were lower than those reported by **Sohair and Riad (2002)** (69%) and higher than those reported by **Silva et al. (2018)** (46.88%) and **Abdelsadek et al. (2020)** (48.04%). Similarly, the results of culture in table 1 agreed with those obtained by **Silva et al. (2018)** (68.75%), but were lower than those reported by **Nasr et al. (2016)** (80%) and higher than those given by **Abdelsadek et al. (2020)** (51.59%). Negative culture results may be due to tuberculous lesions being similar to lesions caused by other organisms, which can be distinguished through histopathological examination, or due to M.bovis being killed by macrophages, or due to unsuccessful abattoir sampling **(Arajo et al. 2005)**.

Furthermore, in this study, 65 isolates were confirmed to be M.bovis through Mpb70 targeting PCR and were tested for antibiotic sensitivity against four anti-tuberculous drugs. Table 2 shows that 25 isolates were resistant to at least one of the first-line anti-TB drugs, accounting for 38.5% of the isolates. This prevalence rate of drug resistance was comparable to that reported by **Franco et al. (2017)** (31.3%), but lower than that reported by **Sechi et al. (2001)** (63.6%) and **Wang et al. (2018)** (77%).

In terms of specific drugs, the total resistance to rifampicin and isoniazid was 32.3% and 30.8%, respectively, which were lower than the resistance rates reported by Abdelsadek et al. (2020) (40.7% for rifampicin and 59.3% for isoniazid) and Sechi et al. (2001) (45.5% for rifampicin and 40.9% for isoniazid). On the other hand, our results were higher than those reported by Franco et al. (2017), where the resistance rates to rifampicin and isoniazid were 3% and 12%, respectively. Additionally, MDR-M.bovis was detected in 14 isolates (21.5%), which is nearly similar to the findings of Sechi et al. (2001) (22.7%), lower than Franco et al. (2017) (16%), and higher than Wang et al. (2018) (64.9%).

In terms of molecular analysis, polymorphism in the 81 base pair hot-spot region of the rpoB gene has been linked to about 95% of rifampicin-resistant strains **(Cavusoglu et al. 2002)**.

Automated DNA sequencing has identified at least 5 mutations in this region **(Ahmad et al. 2002)**. In this study, DNA sequencing identified 3 different mutations, with the most frequent one being H526Y detected in 4 isolates. The other mutations detected were D516V (1/6) and S531L (1/6). These results were comparable to those reported by **Silaigwana et al. (2012)**, who reported a high frequency of the

H526Y mutation (90%). Additionally, **Azmy et al. (2022)** reported a mutation in the rpoB gene involving codon 526 in a phenotypic MDR M.bovis and/or M.africanum isolate in Egypt. While we identified the S531L mutation in one isolate, **Blasquez et al. (1997)** reported that rifampicin resistance was mostly associated with the S531L mutation. In contrast, our results differ from those of **Franco et al. (2017)**, who detected no mutations in 21 M.bovis isolates with phenotypic resistance to rifampicin and isoniazid. Furthermore, **Sechi et al. (2001)** reported the H526Y mutation in 2 M.bovis isolates, while our results showed the detection of the L521P mutation in 60% of the tested M.bovis isolates, and the D516V and S531L mutations were not identified among resistant isolates in the same study. These variations in results could possibly be attributed to the geographical location of the isolates in each study.

The limitation of the current study is that the size of the tested samples is small, however, it emphasizes the significance of BTB on public health, particularly due to the emergence of MDR-M.bovis, and emphasizes the necessity for effective control measures to prevent its spread. Despite the small sample size, the study brings attention to the consequences of bovine tuberculosis (BTB) on public health, particularly with the rise of multidrug-resistant M.bovis strains. This highlights the urgency for implementing effective control measures to mitigate the impact of BTB on both human and animal populations. These control measures should encompass strategies such as improved surveillance, early detection, proper diagnosis and treatment, vaccination programs, and strict biosecurity protocols. By addressing these challenges, we can work towards reducing the prevalence of BTB and protecting public health.

CONCLUSION

The results indicated a high prevalence of bTB and multidrug-resistant *M.bovis* in slaughtered cattle in the study area. To mitigate the public health risk, further large-scale epidemiological investigations are necessary, along with surveys focusing on the detectionof *M.bovis* infection in live animals, particularly through ante-mortem methods. Additionally, it is important to conduct drug resistance screening to identify the extent of resistance in *M.bovis* strains. These measures are crucial for reducing the public health hazard associated with bTB and multidrug-resistant *M.bovis*.

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