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Bacteriological and Molecular studies on *Mycobacterium Bovis* in Cattle, with Special Reference to its Antimicrobial Resistance

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ABSTRACT

In current study a total of 153 tuberculous lesion samples collected from cattle after PM examination (69 from El-Menufia, 47 from El-Sharkia and 37 from El-Qaliobia governorates) were subjected to bacteriological examination. Eighty-three samples showed the AF bacilli in direct smears stained by ZN stain at a percentage of 54.2% while by the culture method, *M. bovis* was isolated from 100 (65.4%) samples. Sixty-five isolates, confirmed to be *M. bovis* by conventional PCR targeting Mpb70, were tested for the sensitivity to anti-tuberculous drugs and 25 isolates showed resistance to at least one tested drug. The overall resistance to EMB was 3.1% and resistance to STR, INH and RIF were 20, 30.8 and 32.3% respectively. MDR was detected in 14 (21.5%) isolates. For DNA sequencing, PCR targeting rpoB gene, conferring rifampicin resistance, was performed on selected 6 isolates and identified three mutations designated as H526Y (4/6), S531L (1/6) and D516V (1/6) and the sequences were submitted to the NCBI GenBank with the accession numbers (OM448574, OM448575, OM448576, OM448577, OM448578 and OM448579). The obtained results are epidemiologically significant and augment the necessity of further investigation on ante-mortem identification of *M. bovis* infection in cattle to minimize public risk.

Key words: Bovine tuberculosis, Multidrug-resistant tuberculosis, Sequencing, Anti-tuberculous drugs, Mycobacterial culture.

INTRODUCTION

Bovine tuberculosis (BTB), chronic granulomatous disease of cattle, is caused mainly by *Mycobacterium bovis* (*M. bovis*), a member of Mycobacterium tuberculosis complex (MTBC) (Romha et al. 2018; Nasr et al. 2021). The disease has significant socio-economic importance because of its adverse effects on animal production, international animal trade as well as its potential for human infection (Mohamed 2020). In Human, tuberculosis (TB) is a global public health problem ranking among the top ten leading killer diseases (WHO 2017). In 2019, the global active TB cases in humans were estimated to be ten million incident cases, among them, 140,000 (range 69,800–235,000) were estimated to be new zoonotic TB cases (1.4%) of which approximately death was reported in 11,400 (8.1%, range 4470–21,600) (WHO 2020). In developing countries, about 10-15% of human TB cases are caused by *M.bovis* (Algammal et al. 2019).

Multidrug-resistant TB (MDR-TB) has been defined as disease caused by *Mycobacterium tuberculosis complex* (MTBC) strains resistant to rifampicin and isoniazid which requires prolonged and more complex administration of alternative treatment regimens (Park et al. 1996). Resistant MTBC strains emergence and spreading, especially rifampicin resistance (RR), multi-drug resistance (MDR) and extensive drug-resistance (XDR), is threatening tuberculosis control. In 2015, about 580,000 cases of MDR/RR-TB have occurred globally resulting in 250,000 deaths (Hoffner 2012). In Egypt, it was estimated that TB incidence is 14,000 of new cases per year with MDR/RR-TB incidence is 2.3%, including MDR among new cases is 14%, while in previously treated cases is 30% (WHO 2018).

Isoniazid (INH) resistance is caused by different mutations affecting different genes (Vilchèze and Jacobs Jr 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 detection 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).

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The aim of this study was directed to isolation of MTBC from animal tissue samples collected after PM examination, confirmation of *M. bovis* in obtained isolates by PCR targeting Mpb70, testing isolates for sensitivity to some anti-tuberculous drugs and exploring rifampicin resistance related mutations in some resistant isolates using DNA sequencing.

MATERIALS AND METHODS

Ethical Approval

The study was not conducted on live animals but on tissue samples collected after slaughter in abattoir. As per CPCSEA guidelines (Pandey and Sharma 2011), a study involving clinical and postmortem samples does not require the approval of the Institute Animal Ethics Committee. Those guidelines were followed in the study (Abdelsadek et al. 2020) which was conducted on postmortem samples. However, all owners of slaughtered cattle have signed a consent form. All the laboratory work was carried out in "Biosafety level 3 lab" at Animal Health Research Institute and all staff are aware of the health concern of *M. bovis*.

Samples

A total of 153 tissue samples, showing tuberculous lesions, were collected from slaughtered positive tuberculin reactors in abattoir after PM examination according to the Egyptian guidelines for inspection of cattle (EOS 517 1986) giving special attention to retropharyngeal, bronchial, mediastinal, mesenteric LNs, Lungs, liver, spleen, and serous membranes.

Isolation and Identification

Tissue samples were prepared and inoculated into modified LJ medium with the instructions already mentioned anywhere else (Marks 1972). The preparation of direct smears from processed samples for staining with Ziehl-Neelsen (ZN) stain as mentioned previously (Kubica 1973).

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

DNA extraction from selected 65 obtained isolates was performed using QIAamp® DNA Mini Kit (Qiagen, Germany) as per manufacturer's guidelines. The PCR was performed using primers targeting mpb70 gene: forward (5'-ACCCTCAACAGCGGTCAGTAC-3') and reverse (5'-TTACGCCGGAGGCATTAGCAC-3'), amplifying a 314 bp product, specific for *M. bovis* (Zhang et al. 2016).

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

The susceptibility to some anti-mycobaterial drugs was carried out on 65 isolates, identified as *M. bovis* and confirmed by Mpb70, following the approved standard (M24A) released by the Clinical and Laboratory Standards Institute (CLSI 2011) for Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes.

PCR for Some Drug Resistant Isolates

PCR was performed on 6 selected isolates showing resistance to rifampicin (M-LN-2, M-LN-9, M-LN-12, S-LN-5, S-LN-7 and Q-LN-8), using primers targeting rpoB gene: forward (5'- GGAGCGGATGACCACCC-3') and reverse (5'- GCGGTACGGCGTTTCGATGAAC -3') amplifying a 350 bp product according to (Siddiqi et al. 2002).

Table 1: Results of bacteriological examination of processed tissue samples

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

Table 2: Result of sensitivity of *M. bovis* isolates (n=65) against antituberculous drugs

Tested antimicrobial agent	Number	%
Isoniazid	45	69.2
Rifampicin	44	67.7
Streptomycin	52	80
Ethambutol	63	96.9

Note: Pan-sensitive isolates were 40 (61.5%).

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

DNA Sequencing

PCR products' purification was carried out by the use of The QIAquick PCR Product extraction kit (Qiagen Inc. Valencia CA). Both directions sequencing using Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer/Applied Biosystems, FC, CA). Centrisep (spin column) was used for purification of the sequencing reaction and the reaction was performed on an Applied Biosystems 3,130 automated DNA Sequencer (ABI, 3,130, USA). Comparing sequences was performed in accordance to (Thompson et al. 1994). Neighbour joining, maximum likelihood and maximum parsimony in MEGA6 were used for phylogenetic analysis (Tamura et al. 2013).

RESULTS

It appears from Table 1 that 54.2 % of tested samples harboured the acid-fast bacilli on microscopic examination of ZN-stained smears while 65.4 % were positive by culture method, and all of the obtained isolates were identified according to the morphological characters, growth rate and biochemical tests, as *M. bovis*. The antibiogram results of sixty-five isolates, confirmed to be *M. bovis* by Mpb70 targeting PCR, revealed that 38.5% of tested isolates showed resistance to at least one of the tested anti-TB drugs as shown in Table 2. In addition, 14 (21.5%) isolates were identified to be MDR-*M. bovis*. Sequencing of PCR products from six *M. bovis* isolates showed rifampicin resistance, revealed 3 nucleotide substitution mutations: D516V, H526Y and S531L as shown in Table 3.

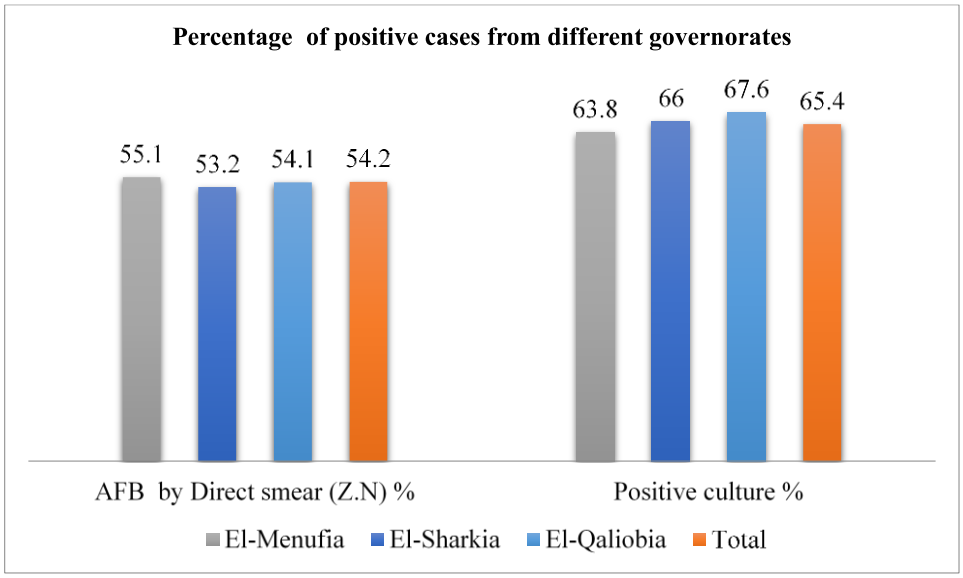

Fig. 1: Results of bacteriological examination of processed tissue samples from different Governorates.

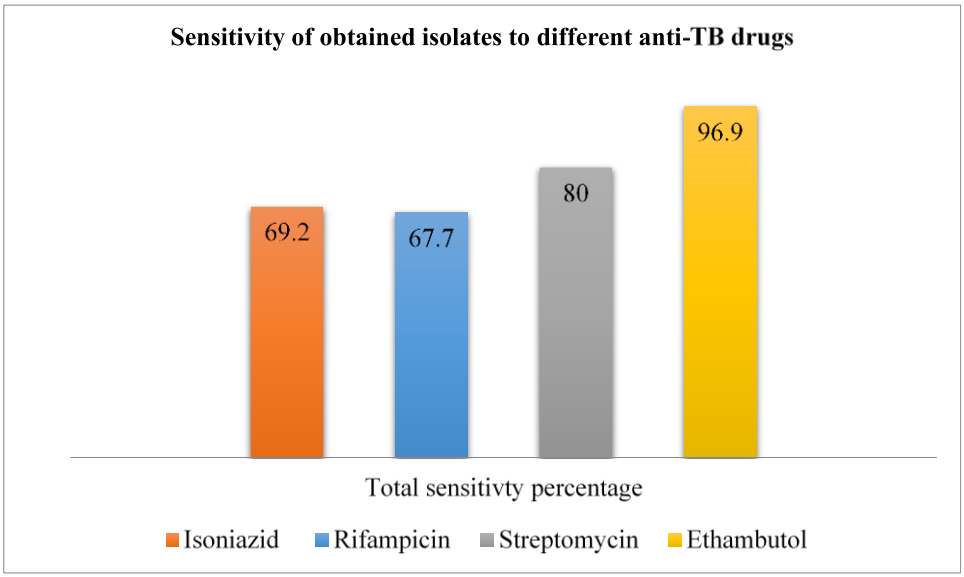

Fig. 2: Sensitivity percentage of obtained isolates to different anti-TB drugs

DISCUSSION

Bovine tuberculosis remains one of the biggest infectious threats to cattle worldwide, making it important to control and prevent outbreaks of MDR-*M. bovis* and its transmission to humans (Franco et al. 2017; Siddique et al. 2022). It's also of great economic impact, which is attributed to a reduction in production, restrictions of animal movement, screening costs, culling of diseased animals, and trade restrictions (Olea-Popelka et al. 2017). From Table 1, it appears that results of direct smear were less than those reported by Sohair and Riad (2002) who detected 69% positive cases but higher than 46.88% and 48.04% reported by Silva et al. (2018) and Abdelsadek et al. (2020). Regarding culture, the obtained results in Table 1 are consistent with those reported by Silva et al. (2018) and Hamed et al. (2021) who detected *M. bovis* in 68.75% and 64.9% of tested samples, respectively, but were less than 80%, 71.4% and 85% reported by Nasr et al. (2016), Mohammed (2017) and Borham et al. (2021), respectively. In contrast, the isolation rate was higher than 51.59% reported by Abdelsadek et al. (2020). Negative culture can result from tuberculous lesions being indistinguishable from lesions caused by other organisms that can be distinguished by histopathological examination, or from *M. bovis* being killed by macrophages (Cousins et al. 2004), or from unsuccessful abattoir sampling (Araújo et al. 2005).

Furthermore, sixty-five isolates were confirmed to be *M. bovis* by Mpb70 targeting PCR and then tested for antibiotic sensitivity against four anti-tuberculous drugs and it appears from Table 2 that 38.5% of tested isolates were resistant to at least one of the first-line anti-TB drugs. This drug resistance prevalence rate was comparable to the 31.3% reported by Franco et al. (2017) and lower than the 63.6% and 77% reported by Sechi et al. (2001) and Wang et al. (2018) but differed from Krajewska-Wędzina et al. (2017) who stated that strains identified as *M. bovis* showed no resistance to INH, RIF, EMB or STR.

On the other hand, the total rifampicin and isoniazid resistance was 32.3% and 30.8%, respectively, which is lower than that reported by Abdelsadek et al. (2020) and Sechi et al. (2001), who reported that resistance to RIF and INH was 40.7%, 45.5% and 59.3%, 40.9%, respectively. But our results were higher than those reported by Franco et al. (2017), where resistance to RIF and INH was 3% and 12%, respectively. Moreover, in the present study, the

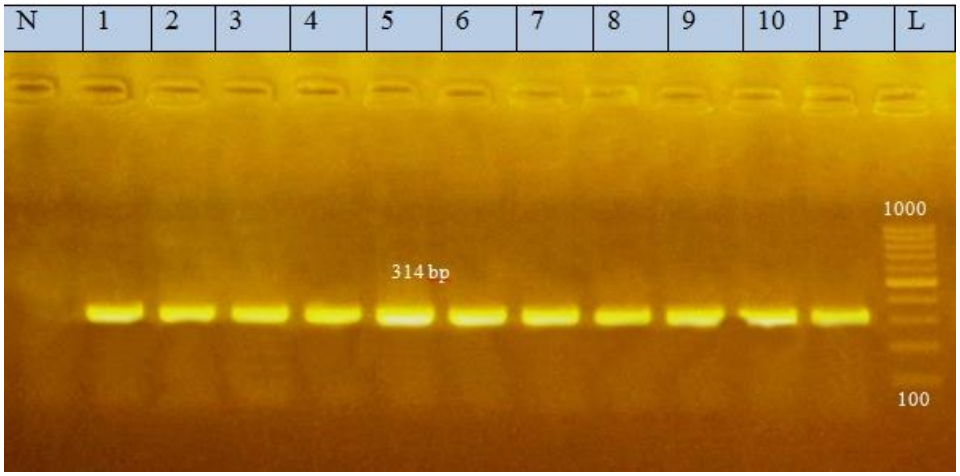

Fig. 3: Agarose gel electrophoresis for PCR amplified products of *M.bovis* isolates
Lane (L): DNA ladder.
Lane (P.): positive control.
Lane (N.): Negative control.
Lane (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10): Positive *M. bovis* isolates with specific amplicon size 314bp.

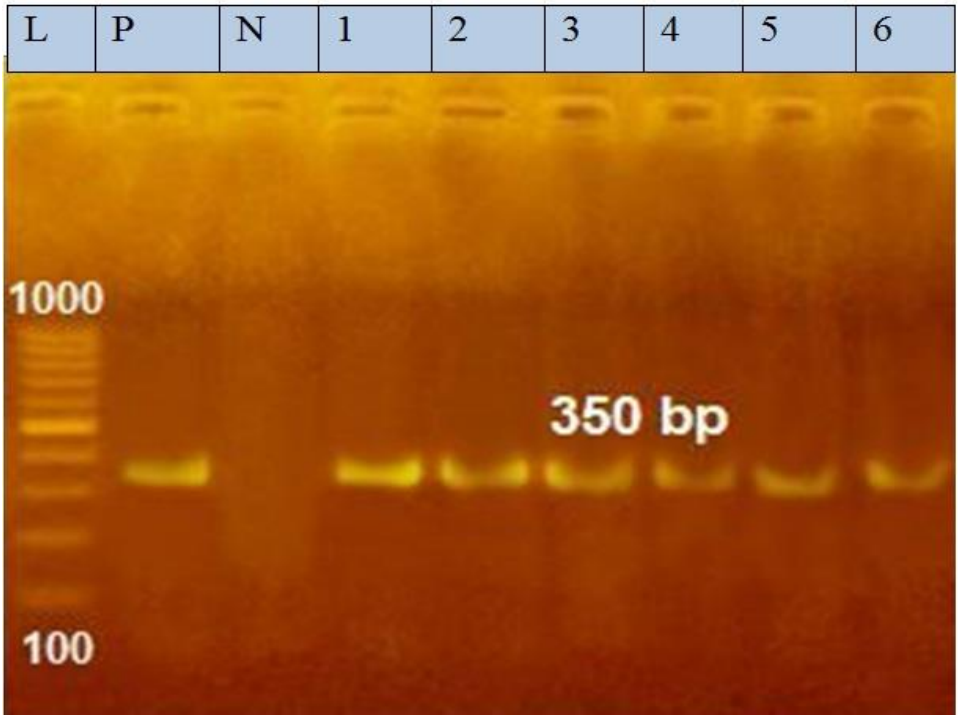

Fig. 4: Agarose gel electrophoresis for PCR amplified products of resistant *M.bovis* isolates.
Lane (L): DNA ladder.
Lane (P.): positive control.
Lane (N.): Negative control.
Lane (1, 2, 3, 4, 5 and 6): positive isolates for *rpo*B resistance gene of rifampicin with specific amplicon size 350 bp.

prevalence of MDR-*M. bovis* was nearly in agreement with Sechi et al. (2001), who detected MDR in 22.7% of isolates, but lower than the 64.9% reported by Wang et al. (2018) and higher than 16 % in a study by Franco et al. (2017). Our results were in contrast to Anne et al. (2019) where none of the tested isolates of bovine origin in their study showed multidrug resistance.

On molecular basis, polymorphism in the 81 base pair hot-spot region for the rpoB gene has been linked to about 95% of the rifampicin resistant strains Cavusoglu et al. (2002). Moreover, automated DNA sequencing identified at least 50 mutations within this region, Ahmad et al. (2002). In this study, DNA sequencing identified 3 different mutations, and the more frequent one was H526Y. This is comparable to Silaigwana et al. (2012) who reported high frequency of the H526Y (90%). In addition, Azmy et al. (2022) reported a mutation of the rpoB gene involving codon 526 in one phenotypic MDR *M. bovis* and/or *M. africanum* isolate in Egypt. Although we identified S531L mutation in one isolate, Blasquez et al. (1997) reported that RIF resistance was associated mostly with S531L mutation in their study. Our results contrast with those obtained by Franco et al. (2017) who detected no mutations in 21 *M. bovis* isolates showing phenotypic resistance to rifampicin and isoniazid. However, Sechi et al. (2001) reported H526Y mutation in 2 *M. bovis* isolates, but our results were different. In the authors' study, L521P mutation was detected in 60% of tested *M. bovis* isolates and D516V and S531L mutation were not identified among resistant isolates in the same study. The variation in results could possibly be attributed to the geographical location of isolates in each study. The present study's limitation is the small size of tested samples, but it highlights the impact of BTB on public health, especially with the emergence of MDR-*M. bovis*, and the need for effective control measures.

Conclusion

The findings demonstrated that bTB was highly prevalent in slaughtered cattle as well as *M. bovis* multidrug resistance in study area. To reduce the public health hazard, further large-scale epidemiological investigations are needed, as well as surveys on ante-mortem detection of *M. bovis* infection, with regards to drug resistance screening.

Author's Contribution

AS and KAA planned the research and experimental design, supervised the work, checked the data analysis and revised the manuscript. SMD collected the samples and performed the laboratory work and data analysis with the help and under supervision of AAE. All authors contributed to the drafting and revision of the manuscript. All authors read and approved the final manuscript

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