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Bacteriological and molecular studies on *Mycobacterium* species in cattle

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For the Ph.D. degree in Veterinary Medical Sciences

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Abstract

In the present study tissue samples showing tuberculous lesions, collected from a total of 153 out of 200 slaughtered positive tuberculin reactors after PM examination, were subjected to bacteriological examination and out of them 83 animals (54.2%) were positive to bTB by microscopic examination of direct smears while by culture on L-J medium, *M.bovis* was isolated from 100 animals (65.4%). Spleen showed the highest positive results by culture technique at percentage of 68.4% followed by lymph node (66.1%), lung (65.1%) then liver (62.9%). Indirect ELISA, using cocktail antigens, on serum collected from the total 200 reactors detected 28% positive bTB cases. The antibiotic susceptibility testing of 65 isolates, confirmed by conventional PCR targeting *Mpb70* as *M.bovis*, revealed resistance to at least one tested drug in 25 (38.5%) isolates of them 14 isolates were MDR. The overall resistance to tested drugs was 3.1%, 20%, 30.8% and 32.2% for EMB, STR, INH and RIF respectively. For exploring mutations associated with RIF resistance, DNA sequencing was performed on 6 *M.bovis* isolates and 3 mutations designated as H526Y (4/6), S531L (1/6) and D516V (1/6) were identified. The sequences were submitted to the NCBI GenBank with the accession numbers (OM448574, OM448575, OM448576, OM448577, OM448578 and OM448579). The obtained results emphasise the health hazard of *M.bovis* and augment the need for further investigation on ante-mortem identification of *M.bovis* infection in cattle to minimize public risk.

Keywords: *M.bovis*, bovine tuberculosis, ELISA, multidrug-resistant tuberculosis, sequencing, anti-tuberculous drugs, mycobacterial culture.

Dedicated with gratitude to

My mother

My father

My wife

My daughters (Laila & Assia)

My brother and sister

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List of ABBREVIATIONS

AFB	Acid Fast Bacilli
Ag85	Antigen 85
Am	Amikacin
BCG	Bacillus Calmette Guerine
bp	Base Pair
bTB	Bovine Tuberculosis
CDC	Centers for Disease Control and Prevention
CFP-10	Culture Filtrate Protein 10
CI	Confidence interval
CIDT	Comparative Intradermal Tuberculin Test
CITT	Comparative Intradermal Tuberculin Test
Cm	Capreomycin
CMI	Cell Mediated Immunity
CSF	Cerebrospinal Fluid
DNA	Deoxy ribonucleic acid
DST	Drug Susceptibility Test
ELISA	Enzyme Linked Immunosorbent Assay
EMB	Ethambutol
ESAT-6	Early Secretory Antigenic Target 6
FAO	Food And Agriculture Organization
GOVs	General Organization of Veterinary Services
ICGA	Immunochromatographic Assay
IELISA	Indirect Enzyme Linked Immunosorbent Assay
IFN-γ	Interferon Gamma
IgG	Immunoglobulin G
IGRA	Interferon Gamma Release Assay
INH	Isoniazid
kDa	Kilo Dalton
Km	Kanamycin
LAMP	Loop-mediated isothermal amplification
L-J	Lowenstein-Jensen medium
L.N	Lymph Node
LPA	Line Probe Assay
MDR	Multidrug Resistance
MIC	Minimal Inhibitory Concentration

MODS	Microscopic Observation Broth Drug Susceptibility
MOTT	Mycobacteria Other Than Tuberculosis
mPCR	Multiplex Polymerase Chain Reaction
MTBC	*Mycobacterium Tuberculosis* Complex
MTC	*Mycobacterium Tuberculosis* Complex
NAATs	Nucleic Acid Amplification Tests
NTM	Non-Tuberculous Mycobacteria
NVL	Non-Visible Lesions
OD	Optical Density
OIE	Office International Des Epizooties
PM	Postmortem
PPD	Purified Protein Derivative
PPD-A	Avian Purified Protein Derivative
PPD-B	Bovine Purified Protein Derivative
PPD-H	Human Purified Protein Dericative
PPD-M	Mammalian Purified Protein Derivative
PZA	Pyrazynamide
RD	Region of Difference
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
rMPB	Recombinant Major Secreted Immunogenic Protein
RR	Rifampicin Resistance
SNPs	Single Nucleotide Polymorphisms
SICCT	Single Intradermal Comparative Cervical Tuberculin Test
SID	Single Intradermal Tuberculin
SIT	The Single Intradermal Tuberculin Test
ST-CF	Short Term Culture Filtrate Antigens
STR	Streptomycin
TLA	Thin Layer Agar
TST	Tuberculin Skin Test
VL	Visible Lesions
VNTR	Variable Number of Tandem Repeat
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR	Extensively Drug-Resistant

Chapter (1)

Introduction

INTRODUCTION

Bovine tuberculosis, a chronic debilitating highly contagious disease affecting cattle, buffaloes and various wild species **(Hardstaff *et al.*, 2013),** is caused by *Mycobacterium bovis* which is one of *Mycobacterium tuberculosis* complex (MTC) **(Romha *et al.*, 2018).** It is characterized by the development of tuberculous lesions which appear most frequently in lymph nodes, lung, liver, spleen, intestines, pleura and peritoneum **(OIE, 2009)**.

The disease has severe economic impacts due to reduction of 10 - 20 % in milk production and weight in cattle, in addition to infertility and condemnation of meat. Without considering the death rate, there is about 10 - 25 % decrease in the efficiency of production in cattle **(Lilenbaum *et al.*, 1999 ; Yingyu *et al.*2009)**, as well as, the great costs of programs for eradication and serious consequences for animals movement and their products **(Rodriguez-Campos *et al.*, 2014).** The annual global losses have been approximated to be 3 billion US dollars costing for 50 million infected cattle **(Maggioli *et al.*, 2015).**

Bovine tuberculosis, as well as its economic impact, is a serious zoonotic problem where man can get infected either by direct exposure to diseased animals or by inhalation of infective droplets (**Perez-lago *et al.*, 2013),** or by ingesting raw milk and meat from diseased animals and their products **(Malama *et al.*, 2013)**. In human, tuberculosis is supposed to be among the leading ten causes of death **(WHO 2017)**, and in developing countries *M.bovis* is responsible for about 10-15% of human cases **(Algammal *et al.*, 2019)**.

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).**

Detection of mycobacterial infections as early diagnosis as possible plays a vital role in control of TB and the application of accurate, reliable and cost effective diagnostic tools are essential for effective detection and subsequent eradication of bTB from cattle population **(Pucken *et al.*, 2017).** Several techniques are used for early diagnosis of bTB infection in cattle which could be expressed in two broad categories namely direct and indirect tests.

The direct tests depend on the detection and identification of the organism in host animal, which primarily relates to the post mortem examination of animals **(Sam *et al*., 2011),** and associated tests to confirm the infection by demonstration of acid fast bacilli by microscopic examination of smears stained with Ziehl–Neelsen stain, the isolation of mycobacteria on selective culture medium and their subsequent identification by culture and biochemical methods **(OIE, 2009)**. Although, the diagnosis based on isolation and identification of *Mycobacterium* is tedious and time consuming **(Cedeno *et al*., 2005)**, it remains the golden standard for tuberculosis diagnosis where its specificity assumed to be very high but the sensitivity is relatively low due to insufficient materials submitted for culture and failure of culture to isolate the organism when it presents at low levels **(Sam *et al*., 2011)**.