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Original Research Article
Veterinary Medicine and The One Health Concept

MOLECULAR CHARACTERIZATION SOME BACTERIAL PATHOGENS CAUSING BOVINE MASTITIS WITH SPECIAL REFERENCE TO *Mycoplasma bovis*

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Abstract: In dairy industry, bovine mastitis is the most prevalent disease, which reduces milk production and causes economic losses. This study was conducted to estimate the prevalence of *Mycoplasma bovis* and some bacteria causing mastitis in dairy farms and partial sequencing of 16SrRNA target genes and Quinolones Resistance Determining Regions (QRDRs) (*gyr*A and *par*C) in *M. bovis* isolates. 370 milk samples were obtained from farms located in villages in Fayoum governorate, Egypt. The examined milk samples (8,91%) were positive for the California mastitis test (CMT). Multiplex RT-PCR was used for the recognition of microorganisms causing mastitis (*Staphylococcus (S.) aureus, Streptococcus* species (spp.), *Escherichia (E.) coli*, and *Mycoplasma (M.) bovis*) from mastitic milk. The results revealed that *E. coli* was the most predominant (84.8%) followed by *S. aureus* (81.8%) while *M. bovis* was the lowest one (51.5%). Mixed infection with two or more mastitic bacterial agents was also identified. All 33 examined mastitic milk samples were diagnosed with mixed infection with *E. coli*, *S. aureus, Streptococcus* spp. and *M. bovis* (36.36%), *E. coli* and *S. aureus* (21.21%), and rephrase *E. coli*, *M. bovis,* and *Streptococcus* spp. (6.06%). The sequence analysis of *M. bovis* 16SrRNA genes illustrated a high similarity of examined isolates to strains previously deposited in the GenBank recovered from the same locality. The *gyr*A amino acids showed no substitution but showed 100% similarity with *M. bovis* isolates worldwide. However, the amino acid sequence of *par*C, showed substitution at positions 2 (Gln to Arg) (CAG >>CGT), 75 (Ile to Ser) (ATT>>AGC), and 79 (Asn to Asp) (AAC>>GAT). Sequence results can lead to the creation of appropriate treatment and control measures for *M. bovis*, while multiplex RT-PCR, can be exploited as a standard diagnostic method for major mastitis pathogens.

Key words: mastitis; multiplex RT- PCR; *M. bovis*; sequence; molecular diagnostics

Introduction

Mastitis is a complicated issue that affects both the quantity and grade of milk produced by milk-producing cattle [1]. In addition to decreased milk production, treatment cost, and disposal of milk, mastitis impacts the sale price of milk and causes animals to be culled [2,3]. Mastitis has various etiologies and is commonly caused by bacterial species [2] such as *S. aureus*, *Streptococcus agalactiae*, *Mycoplasma* spp., *E. coli*, *Klebsiella* spp., Enterobacter spp., *Streptococcus uberis*, *Serratia* spp., *Pseudomonas* spp., and *Proteus* spp. [4]. The dominant bacterial species are *E. coli*, *S. aureus*, and *Streptococcus* species [5]. *E. coli* was recently detected in many mastitis cases in dairy farms [6]. *S. aureus* is a facultative anaerobic gram-positive Staphylococcus bacterium of the udder [7]. It was mentioned that *S. aureus* mastitis is extremely costly for dairy farms and is known for being widespread and chronic [8]. *Streptococcus uberis* is a major mastitis-causing pathogen that is typically categorized as an organism with an

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environmental origin, implying that infection arises from organisms in cows' surroundings [9,10].

M. bovis can cause not only mastitis but also, can be the cause of several disorders in cattle, including pneumonia, arthritis, genital problems, keratoconjunctivitis, and otitis media. It is regarded to be a growing, underappreciated cow pathogen [11]. Mycoplasma mastitis frequency has increased recently, particularly in association with dairy herds [12]. *M. bovis* is the predominant species and also the one that results in the most serious clinical cases [13]. Mastitis costs each cow $159 in annual losses. To reduce these costs promptly, and early diagnosis of single and multiple infection by a suitable test is crucial for the adoption of appropriate treatment and control measures. In comparison to conventional tests, the PCR assay is the best method for detecting infection at suboptimal amounts and at a faster rate [14]. By RT-PCR, the time for the recognition of bacteria directly from mastitis samples could be further reduced. These PCR methods allow the recognition of bacteria within hours. Multiplex PCR is advantageous in the simultaneous recognition of many pathogens with cost-effectiveness and thus could be used for quick diagnosis [15].

In this study, multiplex RT-PCR assays were used to identify microorganisms that cause mastitis in dairy farms (*M. bovis* and other bacteria). The target genes (*M. bovis* 16S rRNA) and QRDRs (*gyr*A and *Par*C) of *M. bovis* were partially sequenced to determine whether they are genetically related to other *M. bovis* isolates, particularly those that cause mastitis in dairy farms.

Material and methods

Study design

370 milk samples (300 cows' milk and 70 buffaloes' milk) during three years in 2019 (100 cows and 30 buffaloes), 2020 (100 cows and 20 buffaloes and 2021 (100 cows and 20 buffaloes) were obtained from three private dairy cattle farms and farmers located in villages in Fayoum governorate, Egypt. The examined animals with a history of being suffered from mastitis and reluctance to antibiotic therapy or the recent replacement of newly purchased animals from local markets without previous examination to *M. bovis* infection. The individual farmers had cows or buffaloes or both. The farmers are mainly keeping their animals in the back-yard of their houses. The back-yard units are connected directly to the owner's home. From a separate quarter of animals, 25 ml of milk samples were obtained in sterile bags under sterile conditions. The samples were transported directly at 4°C to the laboratory for bacteriological examination.

California mastitis test (CMT)

The collected Cows and buffalos' milk were tested for subclinical mastitis by the California test according to [17].

Multiplex RT-PCR

The CMT-positive samples were subjected to multiplex RT-PCR for bacterial-causing mastitis *E. coli, S. aureus, Streptococcus* spp, and *M. bovis.*

Table 1: Nucleotide sequence of primers and probes used in the multiplex RT-PCR assay

Primer/ probe	Sequence 5´- 3´	Reference
M. bovis. F	GAGAATGCTTCAGTATTTTGACGG	[18]
M. *bovis.* R	CAAAAGCAAAATGTTAAATTCAGG	
M. *bovis*-Prob ROX	CAL Fluor Red 610- -CATATATAAGTGAGACTA ACTTATT-BHQ2	
E. coli. F	CGCCTAATCCGCAACGTAAT	[19]
E. coli. R	CGCAGCGTGATCCTGTTTAT	
E. coli-Prob FAM	FAM-TGGCGCAGATGACTGATAAAGCCA-BHQ1	
Strept. F	GTACAGTTGCTTCAGGACGTATC	
Strept. R	ACGTTCGATTTCATCACGTTG	
Strept-Prob HEX	CAL Fluor Orange 560 -ACAATTGGACGAAGGTCTTGCTGGA-BHQ1	
S. aureus. F	TCGAAATTAAATGTTGTCGTGTCTTC	[20]
S. aureus. R	TCATTTTTGACATGRAGAGAAACATC	
S. aureus-Prob FAM	FAM- TCGCGACATTCATTATGCCCAAATTTTTAA-BHQ1	

Table 2: Nucleotides sequence of primers and amplified products for four target genes of *M. bovis*.

(Target Gene)	Primer Sequence (5'-3')	PCR Amplified Product (bp)	Reference
16S ribosomal RNA for ruminant *Mycoplasma*	F: AGACTCCTACGGGAGGCAGCA R: ACTAGCGAT TCCGACTTCATG	1000bp	[22]
M. bovis (16S rRNA)	F: CCTTTTAGATTGGGATAGCGGATG R: CCGTCAAGGTAGCATCATTTCCTAT	360bp	[23]
QRDRs (gyrA)	F: GACGAATCATCTAGCGAG R: GCCTTCTAGCATCAAAGTAGC	531bp	[24]
QRDRs (parC)	F: GAGCAACAGTTAAACGATTTG R: GGCATAACAACTGGCTCTT	488bp	[24]

DNA Extraction:

25ml of milk sample was centrifuged at 4000 g/20 min. Then the supernatant was discarded, and the pellet was cleaned twice with sterile phosphate-buffered saline (PBS). After that, re-suspend the pellet in 200µl of sterile PBS. Consequently, the nucleic acid was extracted using a QIAamp DNA mini kit (Qiagen, Germany, GmbH) according to the manufacturer's instructions. Finally, the purified DNA was stored at −20 °C until the amplification.

PCR amplification:

PCR amplifications were done on the ABI 7500 (Applied Biosystems, Paisley, UK). RT-PCR assays were executed in 20µl containing 10 µl of 2x Sensifast probe No-ROX buffer (Bioline, UK), 3.75 µl water, 0.25µl of each primer (50pmol conc.), and 0.125 µl of each probe (30pmol conc.) (Table 1) and 5µl of DNA template. Reaction mixtures containing water substituted for DNA templates were used as negative controls. thermocycling conditions were set as per the following parameters: 95°C /15 min, 40 cycles of 95 °C/15 s, 50 °C /30 s, and 72 °C/30s.

Mycoplasma bovis isolation and identification

Mycoplasma PPLO agar and broth media were used for the isolation and identification of *M. bovis* as previously described [21]. Colonies were seen using a stereo microscope.

Detection of M. bovis using conventional polymerase chain reaction (PCR)

DNA from a *Mycoplasma* suspension was extracted using a DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions and was conducted to detect members of the Class Mollicutes (16S ribosomal RNA for ruminant Mycoplasma), one specific for *Mycoplasma bovis* used for the identifying of *M. bovis* isolates (16S rRNA *M. bovis*), and the other two, specific for the detection of QRDRs genes (*gyr*A, *par*C) (Table 2).

Gene sequencing for M. bovis

The PCR product of randomly selected one isolate for 16S ribosomal RNA for ruminant *Mycoplasma*, 2 isolates for *M. bovis* 16S, and one isolate for gyrA, and *par*C genes were sequenced by the GATC Company using an Applied Biosystems 3130 genetic analyzer (ABI, USA) by employing forward and reverse primers and combining the old Sanger method with the new 454 technology. The NCBI Blast search was used to verify the sequencing data, and BioEdit software version 7.1.5 was used to compile and edit chromate graphs. Edited sequences of the *M. bovis* isolate were characterized using BLAST n (http://www.ncbi.nlm.nih.gov/BLAST/) for nucleotide analysis or BLAST p for protein analysis. The homology between the isolates and other chosen reference isolates was assessed using the amino acid sequence identity matrix. The phylogenetic tree was created to determine the genetic relatedness of the *Mycoplasma bovis*. By MEGA version 11, the neighbor-joining (NJ) method generated distance-based [25, 26].

Results

Subclinical mastitis by CMT:

The prevalence of mastitic cows' milk in examined samples was 8%, 6%, and 5% during the

years 2019, 2020, and 2021, respectively. While it was 6.7%, 10%, and 10% during the years 2019, 2020, and 2021 respectively in the Buffaloes' milk.

In 2019, 2020, and 2021, subclinical mastitic cows' milk prevalence was 2%, 2%, and 1%, respectively. In contrast, subclinical mastitis in Buffaloes' milk was 6.7%, 5%, and 0%, respectively (Fig.1). However, overall mastitis in cattle was 8%, while in buffaloes it was 12.8%. From all examined milk samples by CMT 33/370 (8.91%) milk samples were mastitic (25 clinical mastitic and 8 subclinical mastitic)

The recovery rate of Mastitis causing Pathogens using multiplex RT-PCR

The most common pathogen found in examined mastitic milk samples was *E. coli* 28/33 (84.8%), followed by *S. aureus* 27/33 (81.8%), then *Streptococcus* 23/33 (69.6%) and *M. bovis* 17/33 (51.5%) (Fig. 2). Interestingly, the incidences of *E. coli* and *M. bovis* in 2019 were higher than 2020 and 2021 (Fig. 3). The mixed infection by *E. coli, S. aureus, Streptococcus* spp., and *M. bovis* recorded the maximum occurrence rate (36.36%) but mixed infection by *E. coli, Streptococcus spp., and M. bovis* was the lowest occurrence rate (6.06%) (Fig. 4).

M. bovis conventional PCR:

All examined samples were confirmed by both 16S rRNA for ruminant *Mycoplasma* which gave a specific band at 1000bp and 16S rRNA specific *for M. bovis* gave 360bp. However, 20 % positive for *gyr*A, and *par*C each gave specific bands at 531bp and 488bp, respectively.

Sequencing of M. bovis 16SrRNA genes and gyrA and Par C genes:

The sequenced genes were uploaded to GenBank under accession no. OP268410 for

16S rRNA for ruminant Mycoplasma, OP268399, and OP268400 for *M. bovis* 16S, OP270479, and OP270480 for *gyr*A, and *par*C.

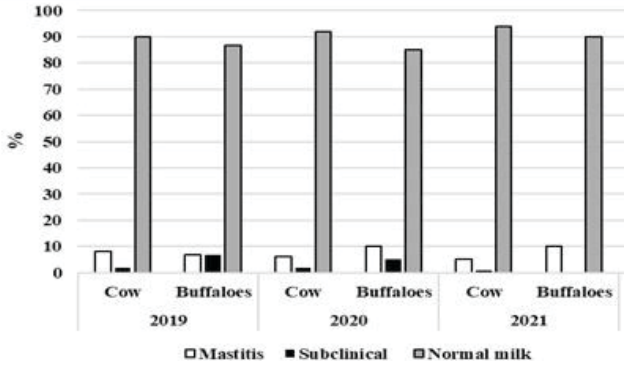


Figure 1: The prevalence of subclinical mastitis by CMT

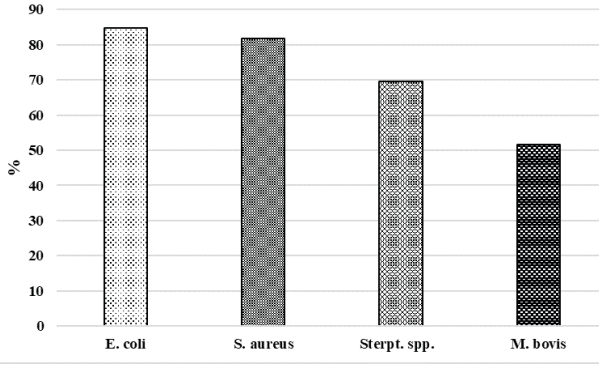
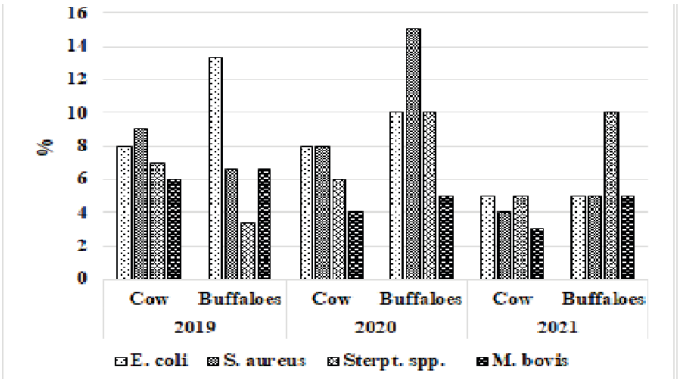


Figure 2: The recovery rate of Mastitis causing Pathogens (*E. coli, S. aureus, Streptococcus* spp., and *M. bovis*) using multiplex RT- PCR. % According to the detected mastitic milk sample (n=33)



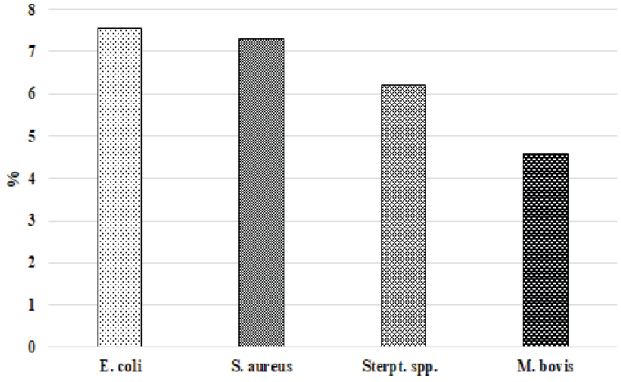


Figure 3: The recovery rate of Mastitis causing Pathogens (*E. coli, S. aureus, Streptococcus* spp., and *M. bovis*) using multiplex RT- PCR; the left panel details result in each year; the right panel the total recovery rate during the study. % According to the total examined milk sample(n=370)

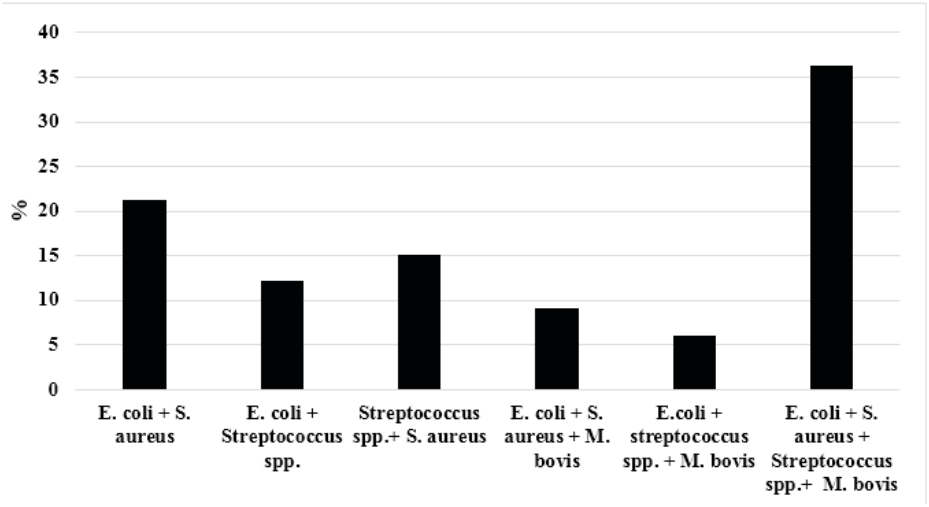


Figure 4: Occurrence of mixed infection and type of organisms detected in examined mastitic milk by RT-PCR. % According to the total examined mastitic milk sample

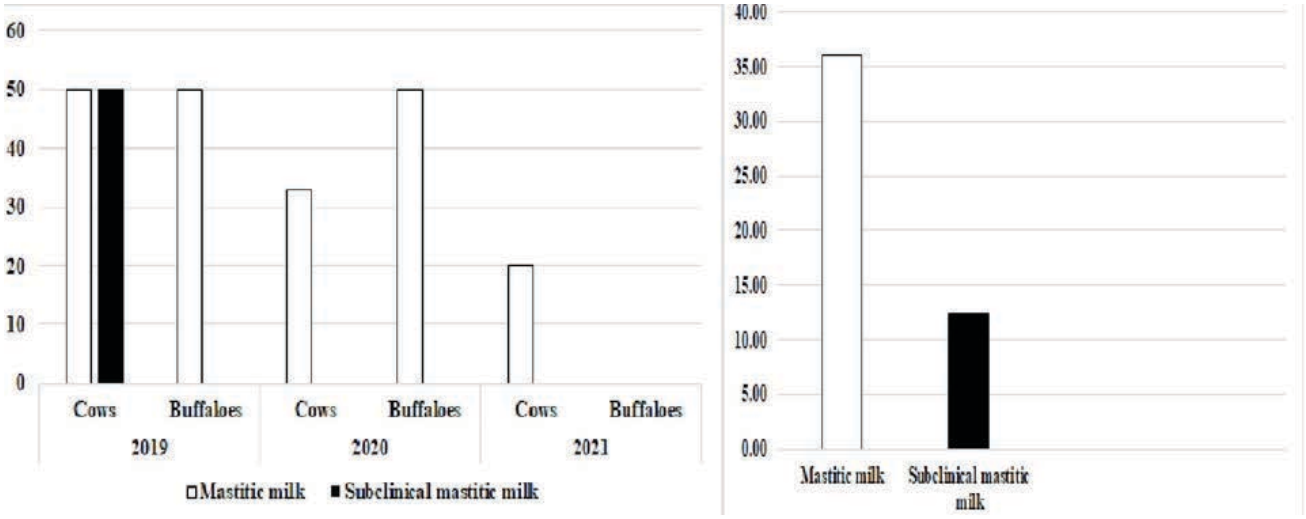


Figure 5: Isolation rate of *M. bovis* in examined mastitic and subclinical mastitic milk samples the left panel showed results each year; the right panel the total recovery rate during the study

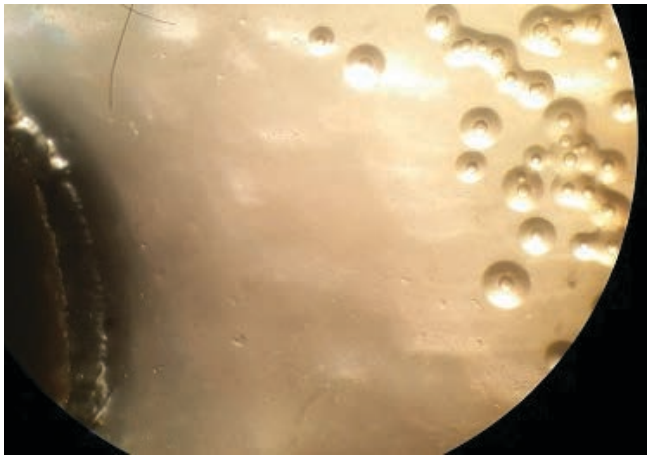


Photo 1: *M.bovis* under stereo microscope

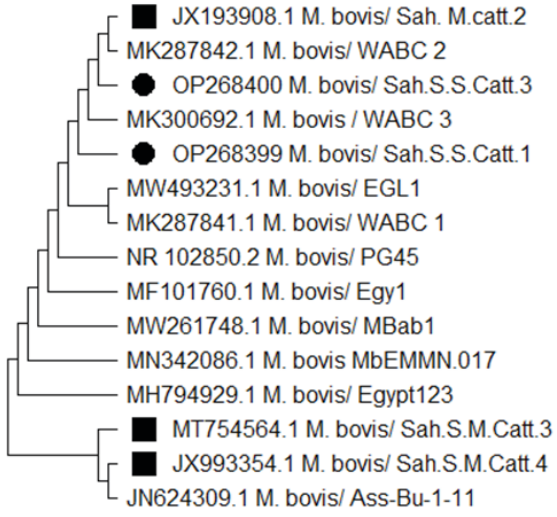


Figure 6: Phylogenetic tree of *Mycoplasma bovis*. 16s gene black circle: this research isolate; black square: previous *Mycoplasma bovis* isolates from the same locality

M. bovis 16S isolates OP268399 and OP268400 showed high nucleotide identity (99% and 97% respectively) with another JX193908 isolate which was isolated from the same locality (Fig. 6).

*M. bovis gyr*A (OP270479.1) showed high nucleotide identity (99%) with other isolates such M. bovis/ CP042939.1, M.bovis/ CP042938.1, and M. bovis/ CP045797.1 (Fig 7 and Table 3). In the current study, *Gyr*A showed no amino acid substitution than *E. coli* K12 (according to *E. coli* numbering).

Mycoplasma bovis *Par*C (OP270480.1 M. bovis/Sah.S.S.par.1) showed high nucleotide identity (100%) with other isolates such M. bovis/ CP042938.1, M. bovis/ CP022596.1, and M. bovis/CP022599.1, M. bovis/ CP045797.1, and M. bovis/ CP045798 (Fig.8 and Table 4). Moreover, *par*C amino acid showed substitutions at positions 2 (Gln to Arg) (CAG >>CGT), 75 (Ile to Ser) (ATT>>AGC), and 79 (Asn to Asp) (AAC>>GAT) (according to E. coli numbering)

Discussion

In milk production, mastitis continues to be one of the most prevalent and expensive illnesses, where the vast majority of clinical and subclinical bovine mastitis occurs. Mastitis caused by *S. aureus*, *S. agalactiae*, and *M. bovis* is now regarded as a serious infectious issue and makes up about 3% of clinical milk submissions. Animal culling remains to be the recommended technique to reduce *M. bovis* mastitis because there are no effective medications or vaccines for the treatment or prevention of this illness, which results in considerable animal replacement expenses for the producer [27].

It was interesting to notice that the prevalence of mastitis in the current study by CMT in cows was (8%) lower than that in buffalo (12.8%) which was in contrast to the previous findings which showed that mastitis in cows' milk more prevalent (32%) than in buffalo milk (22%) [28].

Table 3: Nucleotide identity between *M. bovis gyrA* and other GenBank isolates

Seq->	OP270479.1 M.bovis/ Sah.S.S.Catt.gyr.1	CP042939.1 M. bovis/NADC59	CP042938.1 M. bovis/MJ1	CP045797.1 M. bovis/XBY01	CP022588.1 M. bovis/MJ4	CP022587.1 M. bovis/MJ3	CP019639.1 M. bovis/08M	KR493100.1 M. bovis/MYC22	CP092775.1 M. bovis/L15527	KR493099.1 M. bovis/MYC2	CP092776.1 M. bovis/L15762	CP076229.1 M. bovis/Mb191	CP005933.1 M. bovis/CQ-W70	CP069101.1 M. bovis/Mb304	CP069100.1 M. bovis/Mb300	CP058464.1 M. bovis/TO VK	CP058515.1 M. bovis/VK9	CP062195.1 M. bovis/Tibet-10	E. coli K12	NC_014760.1 M. bovis/PG45
OP270479.1	ID	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP042939.1	100%	ID	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP042938.1	100%	100%	ID	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP045797.1	100%	100%	100%	ID	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP022588.1	100%	100%	100%	100%	ID	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP022587.1	100%	100%	100%	100%	100%	ID	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP019639.1	100%	100%	100%	100%	100%	100%	ID	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
KR493100.1	100%	100%	100%	100%	100%	100%	100%	ID	100%	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP092775.1	100%	100%	100%	100%	100%	100%	100%	100%	ID	100%	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
KR493099.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	ID	99%	100%	100%	100%	100%	100%	100%	100%	95%	95%
CP092776.1	99%	99%	99%	99%	99%	99%	99%	99%	99%	99%	ID	99%	99%	99%	99%	99%	99%	99%	94%	94%
CP076229.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	ID	100%	100%	100%	100%	100%	100%	95%	95%
CP005933.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	ID	100%	100%	100%	100%	100%	95%	95%
CP069101.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	ID	100%	100%	100%	100%	95%	95%
CP069100.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	ID	100%	100%	100%	95%	95%
CP058464.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	ID	100%	100%	95%	95%
CP058515.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	ID	100%	95%	95%
CP062195.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99%	100%	100%	100%	100%	100%	100%	ID	95%	95%
E. coli K12	95%	95%	95%	95%	95%	95%	95%	95%	95%	95%	94%	95%	95%	95%	95%	95%	95%	95%	ID	100%
M. bovis/PG45	95%	95%	95%	95%	95%	95%	95%	95%	95%	95%	94%	95%	95%	95%	95%	95%	95%	95%	100%	ID

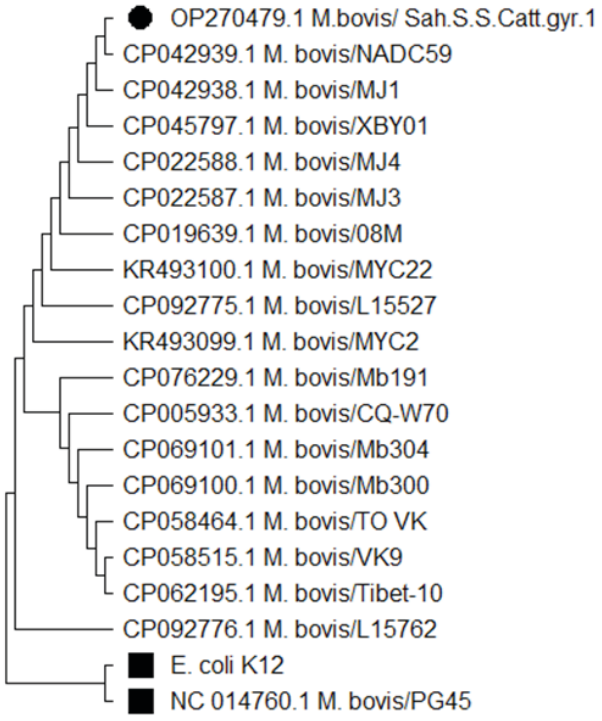


Figure 7: Phylogenetic tree of *M. bovis gyr.* A black circle: this research isolate; black square reference strains

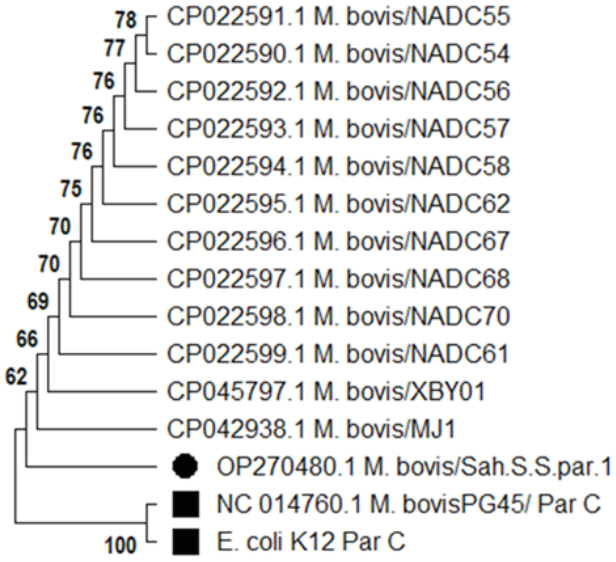


Figure 8: Phylogenetic tree of *Mycoplasma bovis parC*, black circle: this research isolate; black square reference strains

Table 4: Nucleotide identity between *M. bovis gyrA* and other GenBank isolates

Seq->	OP270480.1 M. bovis/Sah.S.S.par.1	CP042938.1 M. bovis/MJ1	CP045797.1 M. bovis/XBY01	CP022599.1 M. bovis/NADC61	CP022598.1 M. bovis/NADC70	CP022597.1 M. bovis/NADC68	CP022596.1 M. bovis/NADC67	CP022595.1 M. bovis/NADC62	CP022594.1 M. bovis/NADC58	CP022593.1 M. bovis/NADC57	CP022592.1 M. bovis/NADC56	CP022591.1 M. bovis/NADC55	CP022590.1 M. bovis/NADC54	NC_014760.1 M. bovisPG45/ Par C	E. coli K12 Par C
OP270480.1	ID	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	74%	73%
CP042938.1	100%	ID	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	74%	73%
CP045797.1	100%	100%	ID	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	74%	73%
CP022599.1	100%	100%	100%	ID	100%	100%	100%	100%	100%	100%	100%	100%	100%	74%	73%
CP022598.1	100%	100%	100%	100%	ID	100%	100%	100%	100%	100%	100%	100%	100%	74%	73%
CP022597.1	100%	100%	100%	100%	100%	ID	100%	100%	100%	100%	100%	100%	100%	74%	73%
CP022596.1	100%	100%	100%	100%	100%	100%	ID	100%	100%	100%	100%	100%	100%	74%	73%
CP022595.1	100%	100%	100%	100%	100%	100%	100%	ID	100%	100%	100%	100%	100%	74%	73%
CP022594.1	100%	100%	100%	100%	100%	100%	100%	100%	ID	100%	100%	100%	100%	74%	73%
CP022593.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	ID	100%	100%	100%	74%	73%
CP022592.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	ID	100%	100%	74%	73%
CP022591.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	ID	100%	74%	73%
CP022590.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	ID	74%	73%
NC_014760.1	74%	74%	74%	74%	74%	74%	74%	74%	74%	74%	74%	74%	74%	ID	99%
E. coli K12 P	73%	73%	73%	73%	73%	73%	73%	73%	73%	73%	73%	73%	73%	99%	ID

The RT-PCR has been demonstrated to be an effective diagnostic method for enhancing bacterial identification and other benefits of reducing throughput time, to enable objective interpretation of data [29]. Additionally, prior research showed that, as compared to bacterial cultures, the real-time PCR technique offers higher sensitivity and specificity for the detection of mastitis pathogens [15]. In this study, the detection rates of *E. coli*, *S. aureus*, *Streptococcus* spp., and *M. bovis* in examined mastitic milk were 84.8%, 81.8%, 69.6%, and 51.5%, respectively. However, the detection rate from Fayoum farms in a prior study was the most frequently identified microbes after isolating *S. aureus* at 37.8% while *S. agalactiae, E. coli, C. pyogenes, E. faecalis,* and *M. bovis* were 13.2%, 12.6%, 8.4%, 5.6 %, and 1.4%, respectively [30].

Moreover, this study's results differed from Katholm *et al.* [31] who mentioned that the detection rate of *Staphylococcus* spp. and *E. coli* was 61% each from all bulk tank milk (BTM) using rt-PCR. Additionally, the recovery rate of *S. aureus* in the current study (81.8%) was higher than reported previously in Egypt 29.1%, 35.9% [19,32], 24.4% [33**]**, Turkey 26.1% [34], but lower than those reported in Denmark 91% [35].

Furthermore, RT-PCR results showed that the most predominant microbial genera were *E. coli* (84.8%) in the investigated mastitic milk samples. While the relatively high frequency of *E. coli* (86.6%) was also reported in Egypt [19]. Based on the results of this study, the incidences of *E. coli*, *S. aureus*, *Streptococcus spp.* and *M. bovis* in examined bovine milk samples in 2019 were higher than recorded in 2021. These may be attributed to insufficient hygienic circumstances, inadequate managemental routines, bad prevention and control measures, contaminated milking equipment and deficient supervision.

In the meantime, *E. coli* was identified in a mixed infection together with *S. aureus* in 21.21% of investigated samples, with *Streptococcus* spp. in 12.12% of investigated samples, together with *S. aureus*, *M. bovis*, and *Streptococcus* spp. in 36.36% of examined samples. These findings were higher than those recorded previously [36]. On the other hand, the occurrence rate of *E. coli* mixed with *S. aureus* and *E. coli* mixed with *Streptococcus* spp. in examined samples was near to the previous study [16].

Comparatively, the recovery rate of *M. bovis* recorded in the present study was 51.5% which was higher than that found in BTM in both Cyprus at 6.25% and Canada at 0.53% [35].

A three-year study on *M. bovis* mastitic infection was carried out at dairy farms in the Fayum Governorate between 2019 and 2021. These herds had previously been certified as *M. bovis* positive, and the prevalence of *M. bovis* in mastitic milk ranged from 50% in 2019 to 20% in 2021. This may be due to the various stages of infection, the variable *M. bovis* strains, the intermittent shedding of the microbe by the infected cows, or the low concentration of *M. bovis* in the tested milk samples, as mentioned by [37] or the application of strict biosafety and biosecurity measures in these farms.

It is known that molecular typing of *Mycoplasma* is extremely helpful for testing dairy farms, clinical samples, and for identifying mollicutes that are difficult to cultivate or that grow slowly. The 16SrRNA group-specific PCR assay amplifies *Mycoplasma* of ruminants and also all other mollicutes species that belong to the genera *Mycoplasma*, *Acholeplasma*, *Ureaplasma*, and *Spiroplasma* but does not amplify sequences of any other prokaryote [38, 39]. In this study we used16srRNA common for Ruminant according to [22] and another one more specific for *Mycoplasma bovis* according to [23].

The molecular typing elucidates the phylogenetic analysis and detects any mutation in the circulating strains. molecular typing and sequencing of *M. bovis* isolates can give additional information about their relationships and evolution [37]. From the sequence of 16sRNAgenes common and specific for *M. bovis* the isolates OP268399 and OP268400 showed high nucleotide identity (99% and 97% respectively) with another JX193908 isolate which was isolated from the aforementioned locality. The *gyr*A and *par*C genes of *M. bovis*, which are linked to Enrofloxacin resistance, were examined for mutations. The phylogenetic tree of *Mycoplasma bovis gyr*A (OP270479.1) and its nucleotides identity showed 100% similarity between our *M. bovis gyr*A and other GenBank isolates circulating worldwide and 99% similarity with CP092776 *M. bovis* strain L15762 which circulates in France.

According to the *E. coli* numbering system, Mycoplasma bovis *Par*C (OP270480.1 M. bovis/ Sah.S.S.par.1) in our investigation displayed

amino acid transpositions at positions 2 (Gln to Arg) (CAG >>CGT), 75 (Ile to Ser) (ATT>>AGC), and 79 (Asn to Asp) (AAC>>GAT), while [40] found four potential mutations (Asp79Asn, Ser80Ile, Ser81Pro, and Asp84Asn/Tyr/Val/ Gly). The result of amino acid transpositions at positions 2 (Gln to Arg) in Mycoplasma bovis *Par*C is the second time to be recorded in Egypt similar to [41] who stated that routine molecular techniques such as DNA sequencing can help in detecting mutation sites As amino acid positions are identified using the traditional *E. coli* numbering system, the newly proposed Epidemiological Cutoff determents (ECOFF) a single mutation in either *Gyr*A (Ser83) or *Par*C (Asp79) [40], *M. bovis* strains were divided into (non-)WT or susceptible/resistant isolates.

Conclusion

Major mastitis pathogens (*M. bovis* and other bacteria) could be detected using multiplex RT-PCR, which can be exploited as a standard diagnostic method.

The phylogenetic analysis of the target genes (*M. bovis* 16S rRNA) showed high genetic relation to isolate from the aforementioned locality; while that of Mycoplasma bovis *gyr*A (OP270479.1) showed 100% similarity with other GenBank isolates circulating worldwide and 99% similarity with *M. bovis* strain circulates in France.

Mycoplasma bovis *Par*C (OP270480.1 M. bovis/Sah.S.S.par.1) displayed, amino acid transpositions at positions 2 (Gln to Arg) (CAG >>CGT) for the second time to be detected in Egypt, and at position 79 (Asn to Asp) (AAC>>GAT) similar to the newly proposed Epidemiological Cutoff determents (ECOFF). We believe that the findings presented in our research may appeal to farmers, veterinary doctors, and researchers and can lead to the creation of appropriate treatment and control measures.

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