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Original Research Article

Molecular Characterization, Virulence, and Antimicrobial Susceptibility of *Mycoplasma bovis* Associated With Chronic Mastitis in Dairy Cows

Key words

antimicrobial susceptibility;
chronic mastitis;
Mycoplasma bovis;
sequence analysis;
virulence genes

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Abstract: *Mycoplasma bovis (M. bovis)* is the most common *Mycoplasma* species, which has a growing impact on the dairy industry. The purpose of this study was to investigate the molecular characterization, virulence, and antibiotic susceptibility of *M. bovis* isolates from dairy cows with chronic mastitis in Egypt. Eighty-four composite milk samples from mastitic cows were aseptically collected from dairy farms in Egypt. Based on microbiological examination and the polymerase chain reaction (PCR) technique targeting the 16S rRNA, 14 (16.7%) of all milk samples were positive for *Mycoplasma* spp. PCR targeting *M. bovis*-specific gene identified six (7.14%) of the 14 mycoplasma isolates as *M. bovis*. PCR assays for different virulence genes showed that all *M. bovis* isolates exhibited the presence of vsp_A gene, while other virulence genes (*uvr*C, *gap*, and *p40* pseudogenes) were determined in only two *M. bovis* isolates (2/6). The 16S rRNA gene phylogenetic analysis demonstrated 100% homology with reference strains of *M. bovis* isolated from different species and locations. When compared to other isolates in the GenBank, the amino acid sequence alignment of our isolate VspA-like protein indicated a distinct mutation. The *in vitro* antimicrobial susceptibility testing of the six *M. bovis* isolates in this study to seven antimicrobial drugs revealed that tilmycosin and tylosin had the lowest minimum inhibitory concentrations (MIC) values (≤1 µg/mL), while danofloxacin, streptomycin and florfenicol had the highest MIC values (<4 µg/mL). Nonetheless, this study showed the virulence of *M. bovis* in dairy cows in Egypt, and macrolides were found to be the most potent compounds *in vitro* against all tested isolates, but further studies in nationwide surveys are needed.

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Introduction

Mycoplasma-caused bovine mastitis is an emergent issue in the dairy industry of various countries. The prevalence of mastitis caused by *Mycoplasma* spp. is increasing, especially in large dairy herds (1-3). This kind of infection causes significant economic losses as it is highly contagious, difficult to detect with conventional culture media, does not respond to antibiotic treatments, could be affect several quarters, produces a significant decrease in milk production with increasing purulent and abnormal secretions, and infected animals are separated or discarded (4). The most frequent *Mycoplasma* species isolated from intramammary infections in cows is *M. bovis*, causing severe clinical cases, followed by other *Mycoplasma* species involving *M. agalactiae, M. californicum, M. bovigenitalium, M. alkalescens*,

and *M. canadense* (5). The major concern associated with *M. bovis* is that causes pneumonia, arthritis, otitis, and reproductive disorders in cattle (5). This pathogen spreads quickly in affected herds and is extremely infectious, making its eradication challenging. The largest obstacle in preventing *M. bovis* infections on farms is represented by asymptomatic carrier animals that spread the disease to other individuals within the population (6).

Laboratory diagnosis is vital to detect *M. bovis* in clinical specimens since there are no specific symptoms associated with *M. bovis* infection; culture is commonly used in combination with polymerase chain reaction (PCR) based detection and identification. Bacterial culture is a gold standard method for detecting infections caused by this pathogen, but it may be laborious, time-consuming, and non-specific, especially if a mixed infection is supposed (7). Compared to bacterial culture, PCR and sequencing-based techniques are very specific but demand a massive amount of effort. For identifying *Mycoplasma* spp. strains in milk samples, PCR is well recognized as a reliable technique. The majority of *Mycoplasma* spp. can be easily distinguished using PCR assays that specifically target the 16S rRNA spacer region (8). Additionally, sequence analysis enables the recognition and differentiation of a number of *Mycoplasma* and *Acholeplasma* species when the *Mycoplasma* 16-23S rDNA and *Acholeplasma* 16-23S rDNA were targeted (9).

Although *M. bovis* is capable of adhering to and invading host cells, produces hydrogen peroxide, avoids phagocytosis, and is resistant to being killed by the alternate complement system, there was limited data on the molecular basis of *Mycoplasma* pathogenicity (10). Variable surface lipoproteins (Vsp) are a class of surface adhesion proteins that exhibit phase and size variation as a result of high frequency configurations of the DNA sequence encoding the Vsp genes, which are crucial for *Mycoplasma* cell attachment (11). Being a part of the DNA repair pathway makes the very stable gene *uvr*C, which produces deoxy-ribodipyrimidine photolyase, necessary for bacterial reproduction (12). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein encoded by the *gap* gene has been demonstrated to encourage an immunological response in beef cattle, which could aid in the development of an effective vaccine (13). An analog of the *Mycoplasma agalactiae* adhesion gene *p40* occurs as a pseudogene in *M. bovis*, where nucleotide sequence analysis found a substantial loss with a frameshift that results in the translation of the protein being prematurely truncated (14).

M. bovis lacks a cell wall and can't produce folic acid, so it is intrinsically resistant to antimicrobials targeting the cell wall e.g., fosfomycin, glycopeptides, or β-lactam antibiotics, sulphonamides, nalidixic acid, trimethoprim, polymixins, and rifampicin, limiting the number of antimicrobial drugs available during treatment may develop chronic mastitis (15). Antimicrobial drugs that affect protein synthesis, such as macrolides and tetracyclines, are frequently utilized for treatment of mycoplasmal infections in animals. There are numerous records on the antibiotic sensitivity of *M. bovis* strains associated with bovine pneumonia, whereas data on strains associated with cattle mastitis is much more limited (16,17). Therefore, identifying changes in this pathogen's susceptibility to antibiotics requires assessing the antimicrobial resistance in *M. bovis* linked to mastitis.

Despite global advances in the study of *M. bovis* virulence factors and pathogenesis mechanisms, a limited number of studies is available from Egypt (1-3). Thus, the objectives of this study were to (i) estimate *M. bovis* prevalence in milk samples from chronic mastitis cases (CM); (ii) determine virulence genes including vsp_A, *uvr*C, *gap*, and *p40* pseudogenes of isolates; (3) perform the phylogenetic analysis of the 16S rRNA and vsp_A sequences in the selected *M. bovis* isolate; and (4) determine the antibiotic susceptibility of isolates using microbroth dilution method.

Materials and methods

Study areas and Ethics

The present study was carried out at 10 dairy cattle farms and 16 small holder farms in three governorates of middle and eastern Delta region of River Nile named Menofia, Dakahlia, and Sharkia.

Ethical approval for collection of samples and clinical data from dairy farms was gained from the Animal Research Ethical Committee at the Faculty of Veterinary Medicine, Mansoura University, Egypt (Code VM.R. 22.11.30). Verbal owner consent for participation in the study was obtained before enrollment.

Clinical examination and sample collection

A total of 84 mastitic cows at 10 dairy farms and 16 small holder farms were investigated. Cows were clinically examined, and a complete clinical report for each animal was constructed. Physical examination of udder, teats, and milk characteristics was conducted. Moreover, duration of the disease and evidence of systemic signs were recorded. In details, 68 samples were obtained from three large loose-housing dairy Holstein cattle herds suffering from chronic mastitis without response to drug treatment in Menofia Governorate and 16 sporadic samples from cattle of mixed breeds in Dakahlia and Sharkia Governorates. The cases had a history of repeated failure of mastitis treatment with a high somatic cell count (SCC) using California Mastitis Test. Following cleaning of teat ends with 70% ethanol, 84 composite milk samples were obtained under aseptic conditions from affected quarters of each animal. Milk samples were uniformly mixed in a 100 mL sterile tube after the first few streams of milk were discarded. During the whole collection and transfer process to the laboratory for

bacteriological examination, samples were preserved on dry ice, and samples were afterwards held at -80 °C until DNA extraction.

Bacteriological analysis

The *Mycoplasma* species isolation from milk samples was carried out as previously described (18). In brief, 100 µl of each milk sample was inoculated into 2.9 mL of pleuropneumonia-like organism (PPLO) broth (Difco, MI, USA) and incubated at 37°C for 72 h. Then, the broth was spread onto PPLO agar plates and incubated under a 10% CO_2 atmosphere at 37°C and inspected for typical *Mycoplasma* colonies every 2 days up to 28 days. The digitonin sensitivity test was performed for differentiation between *Mycoplasma* and *Acholeplasma* genera by filter paper discs saturated with 200 µl of 1.5% (W/V) ethanol solution of digitonin and dehydrated overnight (19). Biochemical characterization using glucose fermentation, arginine deamination, and urea hydrolysis tests, and film and spot formation was carried out for further testing of *Mycoplasma* species (20,21).

Molecular identification of M. bovis and their virulence genes

The genus identification of all strains was established with the 16S rRNA gene by polymerase chain reaction (PCR) test. When the suspicious strains were confirmed as *Mycoplasma* spp., a species-specific PCR was performed for identifying *M. bovis*. Subsequently, the determination of four potential *M. bovis* virulence genes (vsp_A, *uvr*C, *gap*, and *p40* pseudogenes) was also performed by PCR. Briefly, DNA samples were prepared by boiling method from overnight broth cultures as previously described (22). The extracted DNA samples were subjected to PCR amplification by specific oligonucleotide primers and specific cycling conditions (Table 1). PCR amplification was performed in a total reaction volume of 50 µl containing 25 µl Master Mix (Thermoscientific™, K1081), 3 µl target DNA, 1 µl of each forward and reverse primers (10 pmol) and completed to 50 µl by nuclease free molecular biology grade water. Positive control (*M. bovis* reference strain that was previously identified in our laboratory), and negative control were included in the reaction.

Sequencing of 16S rRNA and vsp_A genes

The positive PCR products of one representative strain were sequenced in both directions by Macrogen (South Korea). Using the BLAST and Protein-BLAST search programmes (National Center for Biotechnology Information NCBI" http://www.ncbi.nlm.nih.gov/), the selected *M. bovis* strain (MYEG1) nucleotide and amino acid sequences were compared with those of other strains published in GenBank. The nucleotide sequences obtained during this examination were examined by the BioEdit 7.0.4.1 and Muscle (EMBL's European Bioinformatics Institute, 2020) programs. The subsequent sequences were aligned with those of reference sequences of *M. bovis* utilizing a neighbour-joining analysis of the aligned sequences executed in the program CLC Genomics Workbench 3.

The nucleotide sequences of the *M. bovis* strain (MYEG1), comprising the 16S rRNA and vsp_A genes were deposited in GenBank under accession number OK336362 and OK349676, respectively.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of confirmed *M. bovis* strains for seven antibiotics was performed using PPLO broth supplemented with 0.5% (w/v) sodium pyruvate, 0.5% (w/v) glucose, and 0.005% (w/v) phenol red following the minimum inhibitory concentrations (MIC) guidelines (23) from two different methods, the micro-broth method and Sensititre® plate method. The tested antimicrobial agents were frequently utilized in the treatment of *Mycoplasma* infections. The subsequent antimicrobial substances were investigated: danofloxacin (25%), tulathromycin (10%), doxycycline (50%), florofenicol (10%), oxytetracyclin (20%),

Table 1: Target genes and primer sequences used for PCR amplifications

Target	Specificity	Sequence	Reference
16S rRNA gene	*Mycoplasma* genus	16SmunivF (5'- AGA CTC CTA CGG GAG GCA GCA-3') 16SmunivR (5'- ACT AGC GAT TCC GAC TTC ATG -3')	(8)
Species specific gene	*M. bovis*	MboF (5′CCT TTT AGA TTG GGA TAG CGG ATG-3′) MboR (5′CCG TGA AGG TAG CAT CAT TTC CTA T 3′)	(37)
vsp_A gene		MYBF (5'- CTT GGA TCA GTG GCT TCA TTA GC -3') MYBREV (5'-GTC ATC ATG CGG AAT TCT TGG GT -3')	(8)
uvrC gene		TTACGCAAG bouvrc2-L (5'- TTACGCAAGAGAATGCTTCA-3') M bouvrc2-R (5'-TAGGAAAGCACCCTATTGAT-3')	(40)
gap gene		M.b.gap7(5'-ATAGGAGGATCCAAAAGAGTCGCTATCAATGGTTT TGGACG-3') M.b.gap8 (5'-GGAAATGGTACCTTACTTAGTTAGTTTAGCAAAG TATGTTAATG-3')	(13)
p40 pseudogene		MBO-P40-L (5'-ATGAAAACAAATAGAAAAATAAGTC-3') MBO-P40-R (5'-GTAGCTTTTTCCAATAATTTTCC-3')	(14)

Table 2: Prevalence of Mycoplamsa and *Acholeplasma* in chronic mastitis milk samples (n=84)

Source	Milk Samples No.	*Mycoplasma*			*Acholeplasma*	Total
		M. bovis	Other *Mycoplasma* spp.	Total		
Dairy cattle farms	68	6 (8.8%)	4 (5.9%)	10 (14.7%)	4 (5.9%)	14(20.6%)
Sporadic cases	16	0	4 (25%)	4 (25%)	0	4(25%)
Total	84	6 (7.14%)	8 (9.52%)	14 (16.7%)	4 (4.8%)	18(21.4%)

Table 3: Minimum inhibitory concentrations (µg/mL) of antimicrobials for *Mycoplasma bovis* isolates (n=6) obtained from chronic mastitis

Antimicrobial class	Antimicrobial agents	S* 17	S 26	S 28	S 29	S 30	S 31	MIC ranges
Fluoroquinolones	Danofloxacin	4	4	8	4	8	4	4-8
Tetracycline	Doxycyclin	4	0.312	1.248	4	2	4	0.312-4
Aminoglycosides	Streptomycin	8	0.078	4	0.312	8	8	0.078-8
Phenicols	Florfenicol	8	4	4	8	4	8	4-8
Macrolides	Tulathromycin	4	0.312	1.248	4	4	4	0.312-4
	Tilmycosin	0.039	0.009	0.39	0.009	0.009	0.39	0.009-0.39
	Tylosin	0.009	0.009	1	0.009	1	1	0.009-1

tilmicosin (30%), and tylosin (100%). For each *M. bovis* strain, a sufficient volume of frozen stock culture was thawed and diluted to a concentration of 10^4 color changing units (CCU)/ml as previously described (24) in order to allow for a serial two-fold dilution in fresh broth. In the microbroth assay, the antimicrobials were examined in serial twofold dilutions at concentrations in the range 0.008-16 µg/ml. In Sensititre® plate assay, the antimicrobials were again examined in serial twofold dilutions with concentrations in the range 4-32 µg/ml. Both assays were conducted concurrently so that the outcomes could be readily compared and so that the CCU titration would be useful for both experiments. The endpoint reading of the MIC was the lowest drug concentration that showed no color change in the medium. The final reading was taken between 2-14 days and expressed in µg/ml of active compound. The Clinical and Laboratory Standards Institute's guidelines were used to interpret the results (25). For MIC determination, the susceptibility of *M. bovis* to each drug was tested in triplicate.

Statistical analysis

Statistical analysis of the results by calculation of the ratio was conducted using the SPSS Statistics 17.0 software program.

Results

Clinical signs

The clinically affected cows had a sandy nature, hard quarter (s) (84/84), decreased size (70/84), and reduced milk yield (80/84) without any indication of systemic symptoms.

Bacterial analysis and molecular identification

Out of 84 milk samples, 14 (16.7%) isolates were identified as belonging to the *Mycoplasma* genus based on the culture on PPLO media, the digitonin and PCR tests, while four (4.8%) samples were *Acholeplasma* genus positive (Table 2).

All mycoplasma isolates (n=14) formed typical 'fried-egg' colonies on PPLO medium, were digitonin sensitive, showed film and spot formation and PCR-positive for 16S rRNA gene. Based on the presence of the species-specific gene, only six (7.14%, 6/84) isolates were identified as *M. bovis* from dairy cattle farms. The determination of the virulence genes in *M. bovis* strains showed the presence of vsp_A gene in all strains (6/6), while other virulence genes (*uvr*C, *gap*, and *p40* pseudogenes) were detected in only two *M. bovis* strains (33.3%).

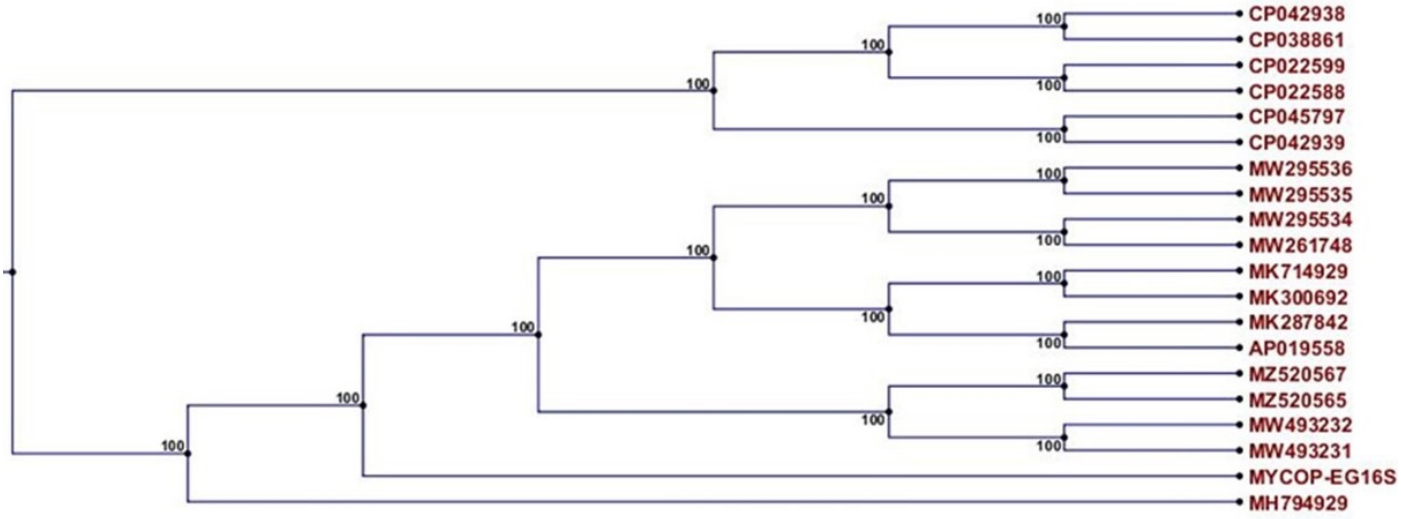

Figure 1: Phylogenetic analysis based on 16S ribosomal RNA gene. This study isolate is designated as MYCOP-EG 16S (OK336362)

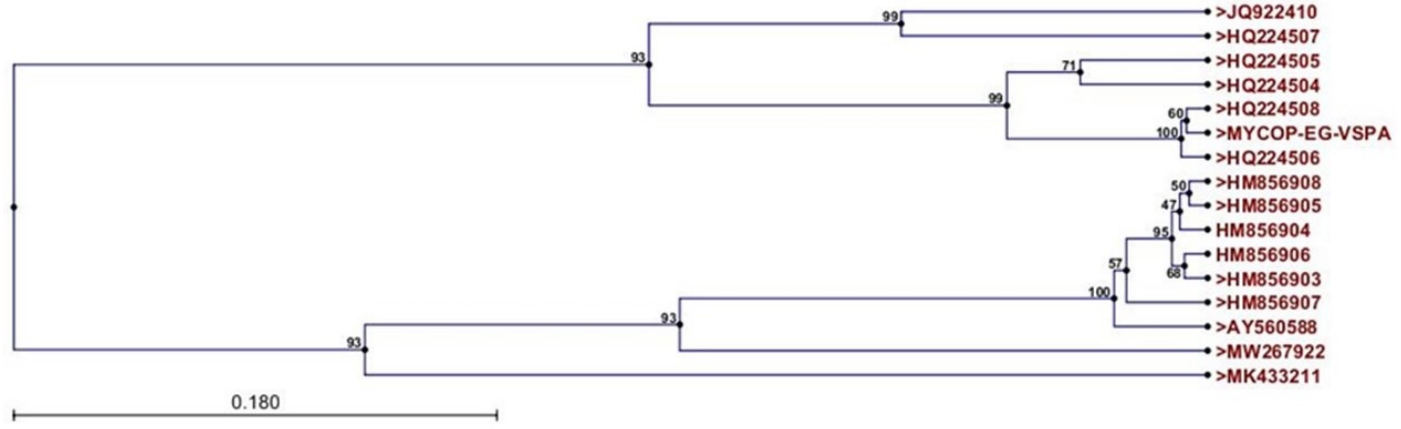

Figure 2: Phylogenetic analysis based on Vsp*A*-like protein gene. This study isolate is designated as MYCOP-EG-VSPA (OK349676)

Molecular and phylogenetic characterization of M. bovis strain

Phylogenetic analysis (Figure 1) showed identical homology with that of *M. bovis* isolated from milk, lung, nasal swab and reproductive organs of bovines, cattle, camel, and poultry from different countries (Egypt, Pakistan, China, and Belgium). Phylogenetic analysis based on VspA-like protein gene (Figure 2) showed high homology of our isolate with that of *M. bovis* isolated from cattle and buffalo milk in Egypt. Sequence alignment of the amino acid sequence of our isolates VspA-like protein responsible for virulence in comparison with other isolates in the GenBank (Figure 3) clearly shows a mutation in amino acid sequence of our isolate's virulence protein even in comparison with other *M. bovis* isolates that were previously isolated from cattle and buffalo milk in Egypt. Phylogenetic analysis based on VspA-like protein sequence (Figure 4) showed a high homology of our isolate with that of *M. bovis* isolated from cattle lung in France (UUA23324) and from cattle and buffalo milk in Egypt (ADT82653 and ADT82657).

Evaluation of the MIC among M. bovis strains

Macrolides were found to be the most active compounds *in vitro* against all examined *M. bovis* isolates from dairy cattle farms. The antibiotic susceptibility results of the Egyptian strains revealed that tilmycosin and tylosin had the lowest MIC values (≤1 µg/mL), while the danofloxacin, streptomycin, and florfenicol had the highest MIC values (<4 µg/mL) for all examined isolates. The isolates were found to be susceptible to tulathromycin and doxycycline with MIC of ≤4 µg/ml (Table 3). The *M. bovis* isolate (S26) was shown to be inhibited by tetracycline, aminoglycosides, and macrolides with low minimum inhibitory concentrations (MIC values). Conversely, strains S17, S30, and S31 were reported to have higher MIC values when most antimicrobials were examined.

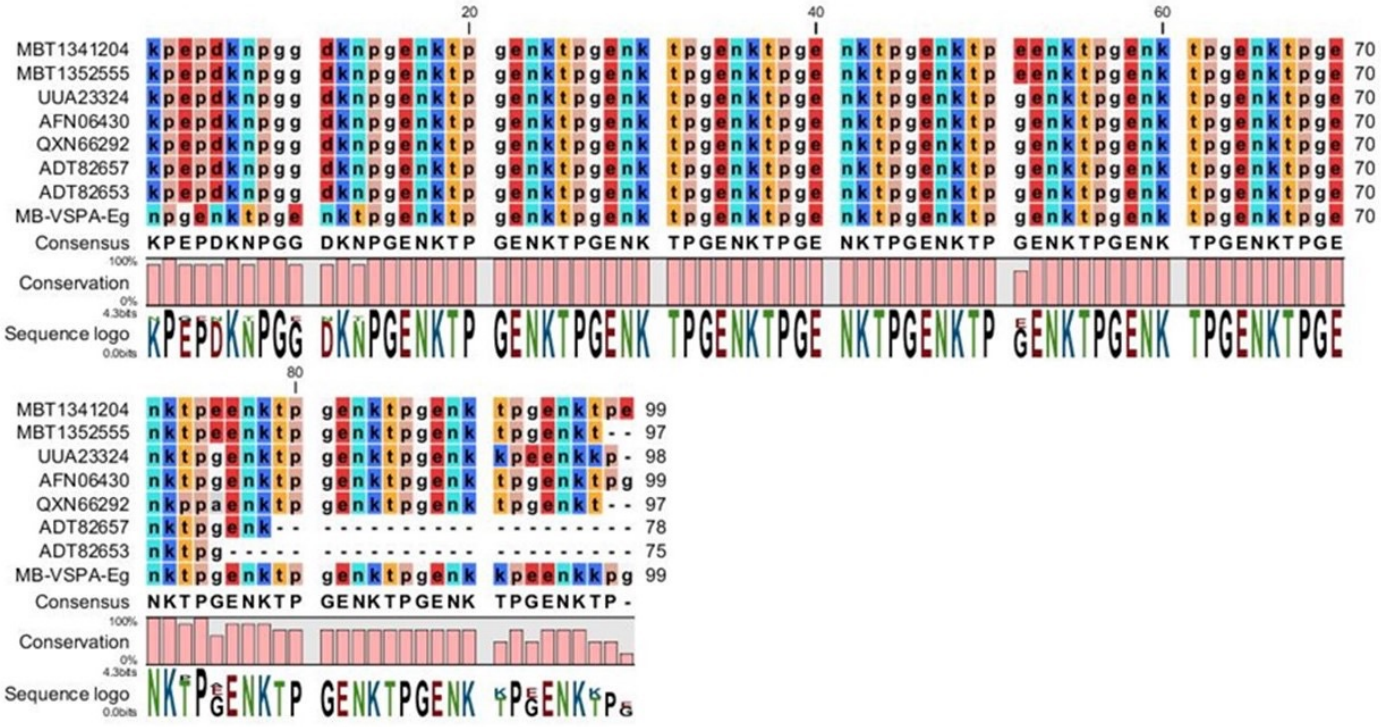

Figure 3: Sequence alignment of the present study *M.bovis* VspA-like protein (UTD45284.1) with the closely related isolates from different sources. Only variable sites are shown with different color

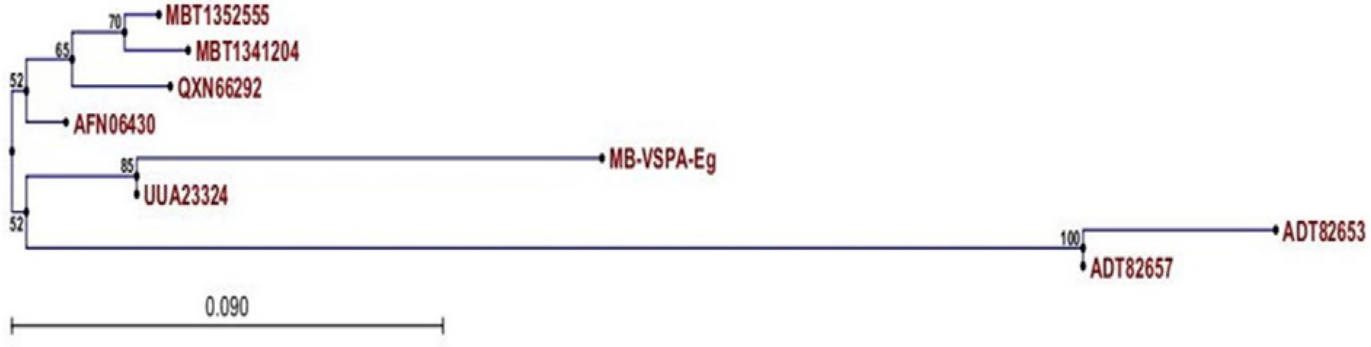

Figure 4: Phylogenetic analysis of the present study M.bovis VspA-like protein (UTD45284.1) with the closely related isolates

Discussion

Mycoplasma, particularly *M. bovis*, is a highly contagious pathogen associated with intramammary infection in the dairy industry, with economic and welfare concerns throughout the world (2). *Mycoplasma* mastitis is thought to be mostly caused by *M. bovis* (26). The ability of *Mycoplasma* species to produce numerous micro-abscesses inside the infected mammary gland causes chronic mastitis, which is extremely costly (27). The current research was assumed to investigate the prevalence of *M. bovis* infections in dairy animals in northern Egypt by both conventional and PCR techniques. The current results showed that 16.7% of the milk samples collected from the dairy farms and sporadic cases were positive for *Mycoplasma* either by conventional culture or PCR technique, while 4.8% of the samples were identified as *Acholeplasma*. Among these, *M. bovis* isolates isolated from dairy farms were identified in percentage of 7.14%. Similarly, Abd El Tawab et al. (1) found relatively low prevalence (8.96%) of *Mycoplasma* including 7.53% of *M. bovis* in Egypt, perhaps as a consequence of variances in farm size (small or large size of the herds), management practices (facility design, pen bedding material, bedding change frequency, and feeding frequency), and hygiene ranking (staff, teat, and equipment hygiene). Also, the low occurrence of *M. bovis* in clinical mastitis samples (8.7%) and bulk tank milk samples (42.3%) was reported in China (3). *Mycoplasma* was previously isolated with a low prevalence (5.3%) from bulk tank and individual cow milk samples with one strain of *M. bovis* in Chile (2). Conversely, the prevalence of *M. bovis* (34.5%) was relatively high in bovine mastitis in Japan (28). The animal cases had a history of

unsuccessful mastitis treatments and high SCC. This issue has persisted for a long time, which may be related to the various ways it might spread, including direct touch, and milking machines (29). Another significant factor for the incidence of *Mycoplasma* mastitis in the examined animals may be intermittent shedding of the pathogen from cows suffering from chronic mastitis.

With the work existing in this investigation, some virulent factors were concerned in the pathogenicity of *M. bovis*. The vsp_A gene was exhibited in all examined *M. bovis* strains (100%), while other virulence genes (*uvr*C, *gap*, and *p40* pseudogenes) were detected in only 33.3% of strains in our study. Diverse repertoires of *vsp* genes have been detected in *M. bovis* (8). These variable surface lipoproteins vsp may be related to *M. bovis* virulence. To distinguish effectively between *M. bovis* and *M. agalactiae*, a PCR based on the DNA repair *uvr*C gene was developed (30). A previous study on *uvr*C gene showed that 19 from 20 of tested *M. bovis* strains isolated from various origins possess 100% identity with the *uvr*C gene sequence (12). Perez-Casal and Prysliak (13) had isolated the *gap* gene of *M. bovis* encoding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Nucleotide sequence analysis of the *p40* * gene in *M. bovis* indicates that *p40* * exists as a pseudogene in *M. bovis* (14).

In the current study the superior PCR technique and partial sequencing of 16S rRNA and vsp_A genes permits for precise diagnosis of infections caused by *Mycoplasma* in cattle and molecular typing of the diverse strains. The sequencing of one representative strain of *M. bovis* MYEG1 strain isolated from a case of chronic mastitis was designated as one of *M. bovis* cluster and showed high homology to other *M. bovis* strains isolated from different countries as published on the gene bank. The sequenced gene of *M. bovis* in the current study was shown to be highly connected genetically to numerous clones from local and global linages, according to our results. The Belgian isolates of *M. bovis* clustered with Israeli, European, and American strains in a different phylogenetic research based on gene sequences of 100 *M. bovis* isolates from dairy, beef, and veal farms over a five-year period (31). In Egypt, El-Tawab et al. (32) demonstrated a close relationship between four sequenced *M. bovis* isolates from respiratory system of cattle. The vsp_A gene sequencing of *M. bovis* isolates from pneumonic lung of camel in Egypt indicated the conserved nature of the vsp_A gene (33) mutation in amino acid sequence in vsp_A protein gene of our strain could lead to variations in antigenicity indices and accordingly these variations might disturb the antigenicity and explain the high virulence of such isolates somewhere *M. bovis* strains could modify the expression of surface antigens and thus to modify the ''antigenic profile'' existing to the host's immune system (20). This is consistent with Eissa et al. (34), who point out the prevalence of circulating *M. bovis* with high diversity power in Egyptian bovine herds that experience mastitis. To better expose its content and dynamics important to disease control methods, the *M. bovis* genome's comprehensive sequencing and assembly could be pursued.

The current research screened the antimicrobial susceptibility profiles of six *M. bovis* strains against the most frequently used antimicrobial agents in the field to control *Mycoplasma* infection in Egyptian dairy farms. The present investigation showed that all isolates had the lowest MICs for tilmicosin and tylosin and highest MICs for danofloxacin, streptomycin, and florfenicol. Tetracyclines and macrolides have always been in the forefront of antibiotic practice for treatment of mycoplasma infection (32). Tilmicosin and tylosin tend to have a bimodal distribution. However, members of the fluoroquinolone (as danofloxacin) and phenicol (as florfenicol) classes of antimicrobials had the lowest MIC_{50} levels across all three decades and were considered effective agents against bovine mycoplasmal disease (3,6,35). The efficiency of tulathromycin in the treatment of *M. bovis* infection was previously detected (35). The MIC_{50} levels for tetracyclines, tilmicosin, and tylosin tartrate were high. It has been reported that tetracycline, spectinomycin, and macrolides have been used to treat diseases associated with *M. bovis*, but resistance and decreased effectiveness have been reported worldwide (35). Tylosin, tilmicosin, tulathromycin, and spectinomycin showed a significant rise in the MIC_{50} while enrofloxacin, danofloxacin, marbofloxacin, and oxytetracycline exhibited a modest increase in France (36). Although mycoplasmas are often susceptible to antibiotics that prevent protein or nucleic acid synthesis, some strains have evolved resistance to these antibiotics by gene mutation or the acquisition of a resistance gene (35). Thus, the prudent use of antimicrobial agents in agriculture and persistence of monitoring of antimicrobial susceptibility of *M. bovis* strains in Egypt should be considered.

In summary, the prevalence of *M. bovis* isolates in the Egyptian dairy farms is potentially a matter of concern. The present study also investigated potential virulence genes and antimicrobial susceptibility profiles of *M. bovis* strains isolated from cattle with chronic mastitis in Egypt. According to the *in vitro* tests that were conducted, macrolides may be the most effective treatment for *M. bovis* infections in Egypt. Furthermore, to prevent and control the introduction and spread of the disease on the farm, priority should be given to farm biosecurity, preventative, and management techniques.

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Molekularna karakterizacija, virulenca in protimikrobna občutljivost z mikoplazmo M. bovis povezanega kroničnega mastitisa pri kravah molznicah

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Izvleček: *Mycoplasma bovis* (*M. bovis*) je najpogostejša vrsta mikoplazme, ki ima vse večji vpliv na mlečno industrijo. Namen te študije je bil molekularno karakterizirati in raziskati virulenco in občutljivost izolatov *M. bovis* za antibiotike, in sicer iz krav molznic s kroničnim mastitisom v Egiptu. Štiriinosemdeset mešanih vzorcev mleka krav z mastitisom je bilo aseptično zbranih na mlečnih farmah v Egiptu. Na podlagi mikrobiološkega pregleda in analize izražanja 16S rRNA z verižno reakcijo s polimerazo (PCR) je bilo 14 (16,7 %) od vseh vzorcev mleka pozitivnih na *Mycoplasma spp.* Z metodo PCR smo šest (7,14 %) od 14 izolatov mikoplazme identificirali kot *M. bovis*. Analize PCR za različne gene virulence so pokazale, da je bil pri vseh izolatih *M. bovis* prisoten gen vsp_A, medtem ko so bili drugi geni virulence (*uvr*C, *gap* in psevdogeni *p40*) izraženi le pri dveh izolatih *M. bovis* (2/6). Filogenetska analiza gena 16S rRNA je pokazala 100-odstotno homolognost z referenčnimi sevi *M. bovis*, izoliranimi iz različnih vrst in lokacij. V primerjavi z drugimi izolati podatkovne zbirke GenBank je poravnava aminokislinskega zaporedja proteina, podobnega VspA, pokazala na značilno mutacijo. In vitro testiranje protimikrobne občutljivosti šestih izolatov *M. bovis* za sedem protimikrobnih zdravil je pokazalo, da sta imela tilmikozin in tilozin najnižje vrednosti minimalnih inhibitornih koncentracij (MIC) (≤1 µg/ml), medtem ko so imeli danofloksacin, streptomicin in florfenikol najvišje vrednosti MIC (<4 µg/ml). Kljub temu da je ta študija pokazala virulenco *M. bovis* pri kravah molznicah v Egiptu, makrolidi pa so se izkazali za najmočnejše spojine proti vsem *in vitro* testiranim izolatom, so potrebne nadaljnje študije v nacionalnih raziskavah.

Ključne besede: : antimikrobna občutljivost; kronični mastitis; *Mycoplasma bovis*; analiza zaporedja; geni virulence