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Comparative Molecular Study of Mycoplasma Bovis Isolates from Egyptian Buffaloes and Cows Suffered from Mastitis

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Abstract: *Mycoplasma bovis* (M. bovis) is widely known to be the most important etiological agent of bovine mastitis. In Egypt, the cases of *Mycoplasma bovis* infection are associated with dairy production losses and are difficult for treatment. The present study was concerned with comparative molecular study of *M. bovis* isolated from cows and buffaloes suffered from mastitis. PCR plus sequencing of variable surface protein A (VspA) gene were used for identification and characterization of eight *M. bovis* isolates in this study. Phylogenetic and sequence analysis showed that, the isolated Egyptian strains were grouped into:-one group which includes Egy-Ca1-10, Egy-Ca2-10, Egy-Bu1-10, Egy-Bu2-10 and Egy-Bu3-10 related to the reference strain; PG45. While Egy-Ca3-10, Egy-Bu4-10 and Egy6-Bu-Dak-12 isolates were existing in a separate group. The present study highlights the presence of circulating *M. bovis* with high diversity power in Egyptian bovine herds. Egy-Ca3-10, Egy-Bu4-10 and Egy6-Bu-Dak-12 revealed conservative residues sequence of 28 amino acids substitutions. However, Egy-Ca3-10 showed 3 amino acids substitutions (T91K, G93E, T97K) that shared by the published strain Sar-1.

Key words: Mycoplasma Bovis · Polymerase Chain Reaction · Variable Surface Protein A · Amino Acids

INTRODUCTION

Mycoplasma bovis is widely known to be the most important etiological agent of various bovine diseases, such as mastitis in cows, pneumonia and arthritis in calves as well as genital disorders [1, 2]. *Mycoplasma bovis* infections tend to be chronic and responsible for considerable economic losses in cattle and milk production [3]. In Egypt (1986) severe outbreak of *M. bovis* mastitis in cows was first reported by Eissa [4]. The disease is characterized by abnormal secretion followed by severe mastitis which did not respond to treatment with antibiotics.

Mycoplasma bovis is called Mycoplasma agalactiae subsp. Bovis [5]. Mycoplasma bovimastitidis or Bovine serotype 5 was named according to Skerman *et al*. [6].

Studies in recent years have shown that populations of several pathogenic mycoplasmal species spontaneously and randomly generate distinct progenies with varied antigenic phenotypes [7, 8]. These antigenic variants may efficiently escape the host immune response and subsequently may play an important role in the chronic nature of mycoplasmal infections [9, 10]. The importance of diversifying the antigenic repertoire of the cell surface in these minute microorganisms is reflected by the fact that, despite their limited genetic material, in mycoplasmas the number of genes that are exclusively dedicated to this purpose is unexpectedly large [9].

The antigenic repertoire of the *M. bovis* cell surface was found to be subjected to rapid changes due to the presence of a set of antigenically and structurally related variable membrane surface lipoproteins designated Vsps. Three members (VspA, VspB and VspC) have so far been characterized [11]. Each Vsp was shown to possess the following features: (i) independent high-frequency phase variation between ON and OFF expression states, (ii) independent high-frequency size variation, (iii) membrane anchorage via the N-terminal domain and a C-terminal region which is surface exposed, (iv) extensive repetitive domains over the full length of the Vsp molecule and (v) regions of shared epitopes [3]. In a more functional aspect, Vsps as a whole or at least some members of the Vsp family are known to be involved in *M. bovis*-adherence to host cells is a prerequisite for colonization and infection [9].

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It was shown that sera collected from animals experimentally or naturally infected with *M. bovis* strongly react with epitopes carried by the Vsps, particularly with those of VspA and VspC, regardless of the clinical manifestations and the geographic location of the natural outbreaks These data taken together with those obtained in the present study that's how the presence of VspA or allelic versions of VspA genes in all field isolates tested so far, provide a solid basis for the development of diagnostic tools based on Vsp antigens [12]. In another study, three Vsps; VspA, VspB and VspC, have been characterized in clonal variants derived from *M. bovis* type strain PG45. Nevertheless, these study data indicated that the recombinant product containing the entire VspA sequence is suitable for the early and late detection of animals infected with *M. bovis*, the Vsp proteins are shown to participate in adhesion to the host cell [13].

The aim of the present work was to study the diversity of *M. bovis* depending upon molecular analysis of VspA gene of *M. bovis* isolates obtained from buffaloes and cows suffering from mastitis.

MATERIALS AND METHODS

Sampling: Milk samples were collected from 59 buffalo and 67 cow dairy herds in 10 Egyptian Governorates which were examined for the detection of Mycoplasma infection. The animals were examined during period from February 2010 to March 2012 including both clinical and subclinical cases.

Isolation and Identification of Mycoplasma Bovis: Milk samples were cultured in broth media [1] and maintained at 37°C for 3-7 days. Biochemical characterization of the isolated purified strains was carried out [14].

Identification of Mycoplasma Bovis by PCR

DNA Extraction: The DNA extraction from the biochemically identified isolates was carried out using QIA Amp ® DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

PCR Amplification: Oligonucleotide primer encoding VspA specific gene for *M. bovis* was used [15]. Primer sequences were as follows: the forward primer; MYBF [5'- CTT GGA TCA GTG GCT TCA TTA GC -3'] and the reverse primer; MYBREV [5'-GTC ATC ATG CGG AAT TCT TGG GT -3']. The primer was prepared by Macrogen Company, South Korea. All the examined M bovis field isolates were positive for VspA gene.

PCR procedure was done according to Alberto *et al.* [15]. The PCR reaction mixture was 50 µl. PCR was performed on a Bio-Rad thermal Cycler (S1000™ Thermal cycler, USA). The amplification was performed by heating the sample for 5 minutes at 94°C, then 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min. at 55°C and extension for 1.5 min at 72°C and a final extension step at 72°C held for 10 min. The analysis of PCR products was performed by using 10 ul of amplified PCR product which was electrophoresed through 1.5% agarose gel and DNA was visualized and photographed by Bio-Rad Gel documentation (Gel Doc ™ XR+ with image Lab ™ Software, USA) after ethidium bromide staining.

Sequencing and Sequence Analysis: The amplified fragments were purified using Gene Jet PCR purification kit; Fermentas (Cat no. KO701). Two GenBank published *Mycoplasma bovis* VspA gene sequences [PG45 (AF396970) and Sar1 (AY560588)] were selected as Reference sequences. Sequencing was performed at Macrogen Company (24, Gasan-dong, Geumchun-gu, Seoul 153-781, Korea) and identification of homologies between nucleotide and amino acid sequences of the *M. bovis* were compared with other strains published on GenBank using BLAST 2.0 and PSI- BLAST search programs, (National Center for Biotechnology Information "NCBI" http:// www.ncbi.nlm.nih.gov/), respectively. The obtained nucleotide sequences comparisons and their multiple alignments with reference *M. bovis* as well as the deduction of amino acid sequences were done using the BioEdit sequence alignment editor [16], CLUSTALX software for multiple sequence alignment [17], ClustalW software for multiple sequence alignment [18], ClustalV [19] and MegAlign (DNASTAR, Lasergene®, Version 7.1.0, USA) [20]. The phylogenetic trees were constructed using MegAlign for tree reconstruction of sequences by Neighbor-joining method based on ClustalW. Bootstrapping values were calculated using a random seeding value of 111 [18]. ClustalV was used when end gaps were faced. Sequence divergence and identity percents were calculated by MegAlign. The structural character of VspA protein sequence was identified by Protean (DNASTAR, Lasergene®, Version 7.1.0. USA).

Table 1: Incidence of Mycoplasma infection in cows and buffaloes presented with clinical and subclinical mastitis

	Clinical herds			Subclinical herds		
Animal	Examined herds	*M. bovis* Positive	%	Examined herds	*M. bovis* Positive	%
Cows	34	12	35.3%	33	4	12.1%
Buff.	22	2	9%	37	5	13.5%
Total	56	14	25%	70	9	12.9%

RESULTS

Isolation and PCR Results: All the examined *M bovis* field isolates were positive for VspA gene where 14 out of 56 (25%) herds were positive for *M. bovis* in both cows and buffaloes dairy herds showing clinical mastitis, while 9 herds out of 70 (12.9%) were positive in subclinical herds (Table 1).

Sequence Results: The positive PCR products of 8 *Mycoplasma bovis* field isolates were sequenced successfully for VspA gene. Sequencing of the PCR products of VspA gene were conducted in both directions and a consensus sequence of 421 bp was used for nucleotide and deduced amino acid analysis. The original sequence was trimmed to remove ambiguous nucleotide sequences usually exist in the beginning of the sequencing reaction. Eight VspA sequences were submitted to GenBank database (Table 2).

VspA Nucleotide Sequence Analysis: Analysis of VspA gene nucleotide (nt.) sequence of 5 *M. bovis* isolates; Egy-Ca1-10, Egy-Ca2-10, Egy-Bu1-10, Egy-Bu2-10 and Egy-Bu3-10 isolates, showed close similarity between each other (97.3 up-to 98.6%). Wherever, the other 3 isolates; Egy-Ca3-10, Egy-Bu4-10 and Egy6-Bu-Dak-12 showed close similarity between each other (93.1 up-to 97.5%) and alternatively, were less similar (below 78%) when compared to above 5 isolates (Fig. 1).

Table 2: The sequence results and the accession numbers of the strains submitted to GenBank database

Host	Designation	Accession No.
Cow	EgyCa1-10	HM856903
	EgyCa2-10	HM856904
	EgyCa3-10	HQ224504
Buffalo	EgyBu1-10	HM856905
	EgyBu2-10	HM856906
	EgyBu3-10	HM856907
	EgyBu4-10	HQ224507
	EgyBu6Dak-12	JQ9224101

While VspA gene nt. sequence of the published strains; PG45 and Sar1 revealed that they are more similar to mentioned above 5 isolates; Egy-Ca1-10, Egy-Ca2-10, Egy-Bu1-10, Egy-Bu2-10 and Egy-Bu3-10 (nt. identity was 93.5% up-to 97%) than the late 3 isolates; Egy-Ca3-10, Egy-Bu4-10 and Egy6-Bu-Dak-12 (nt. identity was 71.2% up-to 81.2%).

VspA Amino Acids Sequence Analysis: A consensus of about 100 amino acids (a.a.) was used for sequence analysis of the deduced a.a. sequences of the consensus (Fig. 2).

Analysis of Egy-Ca2-10, Egy-Bu1-10 and Egy-Bu3-10 sequences showed that they are the same strain (100% a.a. identity) and were 100% similar to the reference strain; PG45. Also, the Egy-Ca1-10 and Egy-Bu2-10 were identical (100% a.a. identity) when compared with each other and showed very high similarity (99%)

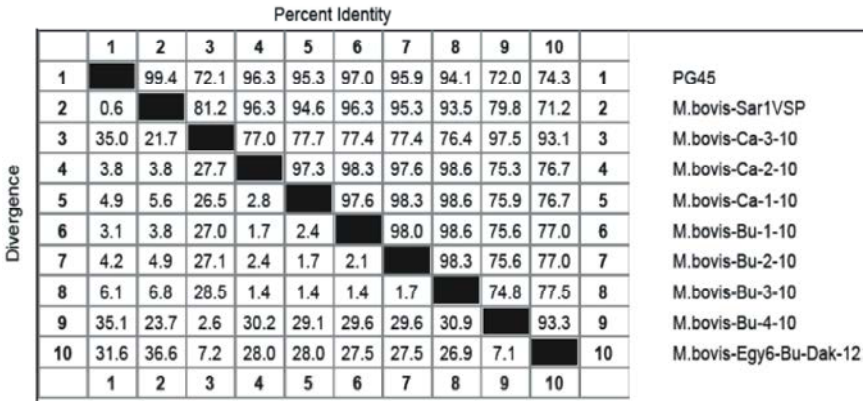

Fig. 1: Percent of identity of nucleotide sequence of VspA gene.

Percent Identity

	1	2	3	4	5	6	7	8	9	10		
1		94.9	71.1	100.0	99.0	100.0	99.0	100.0	74.5	74.5	1	PG45
2	5.3		72.2	94.9	93.9	94.9	93.9	94.9	69.4	69.4	2	Sar1VSP
3	36.4	34.8		71.1	71.1	71.1	71.1	71.1	97.0	97.0	3	Ca-3-10
4	0.0	5.3	36.4		99.0	100.0	99.0	100.0	74.5	74.5	4	Ca-2-10
5	1.0	6.4	36.4	1.0		99.0	100.0	99.0	74.5	74.5	5	Ca-1-10
6	0.0	5.3	36.4	0.0	1.0		99.0	100.0	74.5	74.5	6	Bu-1-10
7	1.0	6.4	36.4	1.0	0.0	1.0		99.0	74.5	74.5	7	Bu-2-10
8	0.0	5.3	36.4	0.0	1.0	0.0	1.0		74.5	74.5	8	Bu-3-10
9	31.2	39.3	3.1	31.2	31.2	31.2	31.2	31.2		100.0	9	Bu-4-10
10	31.2	39.3	3.1	31.2	31.2	31.2	31.2	31.2	0.0		10	Egy6-Bu-Dak-12
	1	2	3	4	5	6	7	8	9	10		

Divergence

Fig. 2: Percent of identity of amino acids sequence of VspA gene

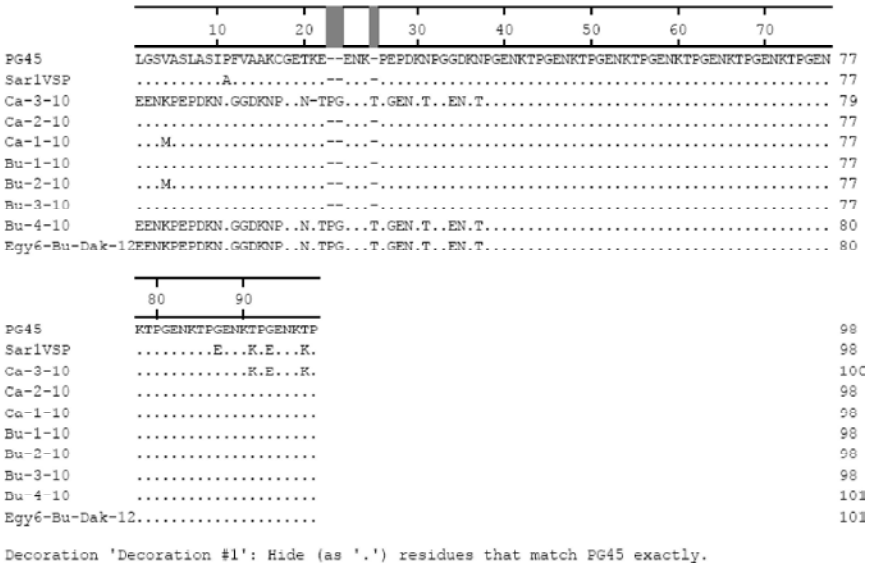

Fig. 3: ClustalW multiple sequence alignment of the deduced amino acid sequences of VspA gene.

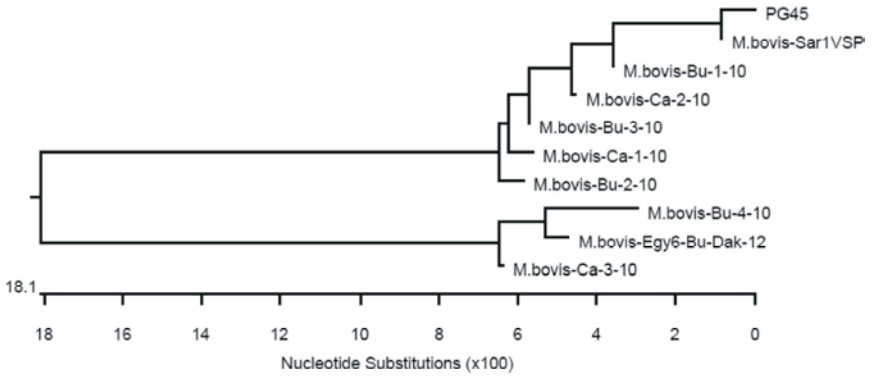

Fig. 4: Phylogenetic tree of nucleotide sequence of VspA gene

when compared to Egy-Ca2-10, Egy-Bu1-10 and Egy-Bu3-10 as well as PG45. When Egy-Bu4-10 and Egy6-Bu-Dak-12 were compared together, 100% homology was obtained. The Egy-Ca3-10 sequence demonstrated closed similarity (97%) with Egy-Bu4-10 and Egy6-Bu-Dak-12, although it was less similar when compared to other sequences (around 72%).

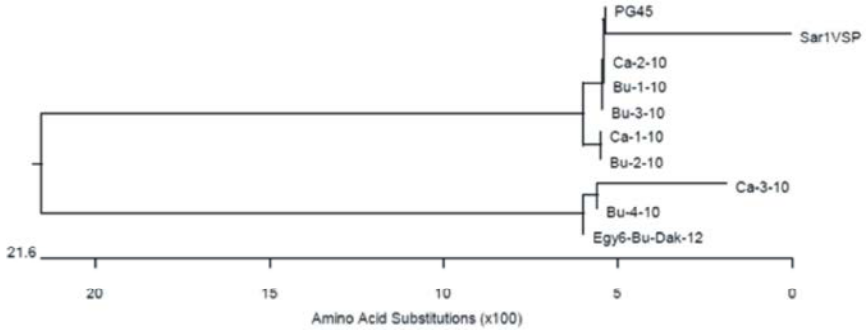

Fig. 5: Phylogenetic tree of amino acids sequence of VspA gene

Amino acids sequence analysis of Egy-Ca1-10 and Egy-Bu2-10 VspA in comparison with selected reference strain PG45 and related isolates; Egy-Ca2-10, Egy-Bu1-10 and Egy-Bu3-10 (Fig. 3), showed a single unique a.a substitution (V4M). On the other hand, Egy-Ca3-10, Egy-Bu4-10 and Egy6-Bu-Dak-12 revealed conservative residues sequence of amino acids substitutions (28 a.a.). However, Egy-Ca3-10 showed 3 a.a. substitutions (T91K, G93E, T97K) that shared by the published strain Sar-1.

Phylogenetic Analysis: The nt. phylogenetic tree of the Egyptian isolates and other reference strains of *M. bovis* VspA revealed that these Egyptian strains are isolating in 2 groups. First group included Egy-Ca1-10, Egy-Ca2-10, Egy-Bu1-10, Egy-Bu2-10 and Egy-Bu3-10 with the reference strain PG45 in same cluster but in different branches. While Egy-Ca3-10, Egy-Bu4-10 and Egy6-Bu-Dak-12 isolates were existing in a separate group (Fig. 4 and 5).

DISCUSSION

Mycoplasma bovis causes major economic losses in dairy industry, inducing chronic disease problems and drop of milk production due to mastitis [21]. In Egypt mastitis has been and still implicated as one of the major disease problem in dairy farms and dairy industry.

Mycoplasma bovis isolates associated with the diseased cases are showing antigenic diversity [3]. The antigenic variants may efficiently escape the host immune response and subsequently may play an important role in the chronic nature of mycoplasma infections [9, 22]. The success of any control programs depend on accurate and timely diagnosis of infected flocks. Therefore, diagnostic assays with high sensitivity, specificity and fast turnaround time are required for the screening of *M. bovis* in bovine herds.

The role of *M. bovis* variable proteins; Vsps, in phenotypic variation as well as pathogenicity has been verified in many studies [3, 7, 12, 14, 23-26]. In particular VspA protein is carrying antigenic epitopes that can be used for rapid identification of *M. bovis* infection [13, 14]. PCR and sequence analysis of this variable gene (VspA) could offer a potential tool for epidemiological and highly discriminating taxonomic studies, such as investigations below the species level [15]. The PCR method has been used to identify *M. bovis* strains with high sensitivity and accuracy [27]. Recently, VspA sequence analysis has been developed to be used for characterization of Mycoplasma strains [14]. Due to the VspA impact in the *M. bovis* structure (as mentioned above), the amino acids changes in this protein could be antigenically and discriminatively significant. Therefore, we used VspA gene sequences for molecular characterization of the isolated strains in this study.

The present study was concerned with detection of *M. bovis* infection in cow's and buffalo's herds. According to isolation and PCR results, *Mycoplasma bovis* mastitis is the most common species isolated from dairy cow and buffalo, where *M. bovis* were isolated from 25% of the clinical cases and 13.5% of the subclinical cases. In USA, Fox and Gay, [28] mentioned that Mycoplasma mastitis appears to be an emerging problem with approximately 10-20% of cow dairy herds affected with *Mycoplasma bovis*. In our study, the majority of cows' isolates was from clinical cases (35.3%) rather than subclinical cases (only 12%), on contrast in case of buffaloes, the *M. bovis* isolates from the clinical cases were less (9%, Table 1). In the current molecular study, we established presence of eight field *M. bovis* isolates: three in cow's herds and five from buffalo's herds (Table 1).

Nevertheless the phylogenetic and sequence analysis showed that there are no specific strains for cow and others for buffalo. Where, similar strains were isolated from both cow and buffalo; cattle strains (Egy-Ca1-10 and

Egy-Ca2-10) and buffalo strains (Egy-Bu1-10, Egy-Bu2-10 and Egy-Bu3-10) were identical or approximately identical. Moreover, two groups of Egyptian isolates of *M. bovis* were obtained in this study. The first, classical group, that are related to the reference strain PG45; Egy-Ca1-10, Egy-Ca2-10, Egy-Bu1-10, Egy-Bu2-10 and Egy-Bu3-10 and the second; Egy-Ca3-10, Egy-Bu4-10 and Egy6-Bu-Dak-12 which were quite different genetically and phenotypically from the published reference strains. These differences could be attributed to the 28 a.a substitutions which exist in isolates of second group. These a.a. mutations have leaded to changes in antigenicity indices as described by Protean® software (data not shown) and consequently these changes could affect the antigenicity of such isolates where *M. bovis* strains can change the expression of surface antigens and thereby to alter the ‘‘antigenic profile’’ presented to the host’s immune system [14].

In conclusion, the present study highlights the presence of circulating *M. bovis* with high diversity power in Egyptian bovine herds. We propose reviewing the current diagnostic tools to include Vsp genes to investigate antigenic diversity of the *M. bovis* in Egypt. We also propose broadening the base of screening for herds with sub-clinical problems.

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