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RESEARCH ARTICLE

Genotyping of *Mycoplasma bovis* Isolated from Cattle Suffering from Respiratory Manifestation in Menofia Province, Egypt

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Mycoplasma bovis in cattle may cause economic losses in cattle farms. Bovine mycoplasmosis is endemic in Egypt. The aim of the current study to determine the occurrence and molecular characterization of *M. bovis* strains recovered from cattle in Egypt. *M. bovis* was isolated by standard methods from nasal swabs, oral and conjunctival swabs of 200 diseased calves with percentages of 40, 15 and 20%, respectively. The examined *M. bovis* isolates were PCR positive to amplified fragment size 1626 bp of *uvr*C gene, 1007 bp of *gap*A gene and 797 bp of p40 pseudogene. Sequence analysis of *uvr*C gene of the field isolates showed (95.3%) similarity when compared with each other and (100%) identity with *M. bovis* reference strain (PG45) and the field strains on GenBank. Analysis of *gap*A gene of *M. bovis* isolates (Egy-8-Fa-14 and Egy-9-DK-14) showed (100%) identity between each other and (98.2%) identity with the reference strain (PG45). Our isolates showed (98.9% up to 100%) identity when compared with international field strains in GenBank. Concerning analysis of p40 pseudogene our field isolates showed (97.5%) identity when compared with each other, while (Egy-12-Fa-14) showed (99.8%) similarity with both *M. bovis* PG45 reference and field strains on GenBank. In conclusion *M. bovis* is circulating in bronchopneumonic calves in Egypt. This is the first record in Egypt to investigate some *M. bovis* genes by nucleotide sequence analysis.

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INTRODUCTION

M. bovis is a common cause of acute respiratory disease mainly affected young calves (Maunsell *et al.,* 2011). Bovine respiratory diseases BRD is a polymicrobial syndrome responsible for high mortality rates in calves caused by variety causes including *M. bovis* (Register *et al.,* 2015). Infectious agents engaged in bovine respiratory disease comprise viruses, bacteria, Mycoplasma and chlamydia. Many viruses have been implicated in respiratory disease in cattle, acting with bacteria to produce serious pneumonia and huge neonate losses (Hartel *et al.,* 2004**)**. In infected cattle cases, *M. bovis* is commonly detected with different micro-organisms in the severe lung lesions (Timsit *et al.,* 2012). *M. bovis* is frequently pathogen causing several clinical diseases as bovine mastitis, otitis media, polyarthritis and gynecological problems (Suleman *et al*., 2016). Different microorganisms were identified in association with *M. bovis* in several diseases outbreaks (Booker *et al.,* 2008). The control of such global problem in dairy farms should include culling of infected animals, prohibit importation from endemic areas, interesting in hygienic sanitary practices as well as removal of predisposing stressful conditions (Heuvelink *et al*., 2016). Phylogenetic and sequence analysis literature about *M. bovis* is lacking in Egypt. Molecular studies of Egyptian *M. bovis* strains will supply knowledge about the status of the molecular diversity of circulating *M. bovis* in Egyptian cattle and help in planning strategy for disease control and/or restriction of disease dissemination through animal transportation. Moreover, the *uvr*C gene, which is part of the excision DNA-repair system uvr ABC, has been shown to be highly conserved and therefore may prove useful diagnostic tool for *M. bovis* strains identification (Hemant*et al.,* 2015). The aim of the current study was to genetically characterize some circulating *M. bovis* strains in young calves suffering from pneumonia in Egypt.

MATERIALS AND METHODS

Sampling: The current work was carried out in a dairy farm of 850 cows in the desert road of Cairo-Alexandria in the period from October 2015 to March 2016. Two-hundered calves of (1-6) months age of Holstein breed, suffering from respiratory manifestation (nasal discharge, coughing, fever, dyspnea and abnormal respiratory sounds in lung auscultation) in Menofia Province, Egypt. Calves were examined and monitored during this period for the detection of Mycoplasma. Nasal, oral and conjunctival swabs (200, each) were collected.

Mycoplasma isolation and identification: Swabs were immersed in 2 ml of Mycoplasma broth medium (Thermo Fisher Scientific Inc. Oxoid Inc, Waltham, MA) and cultured at 37°C. On the second day of incubation, when the slight color change of the broth media occurred, the cultures were inoculated onto Mycoplasma agar media (Thermo Fisher Scientific Inc; Oxoid Inc) and were incubated at 37°C and 5% CO2 for 3 days, until visible colonies appeared as minute transparent, colonies and microscopically as fried egg colonies. *M. bovis* isolation and identification were performed by standard culture and identification methods (OIE, 2012).

Detection of 3 genes of *M. bovis* (*uvr*C, *gap*A, p40) by PCR DNA Extraction: The DNA extraction from the broth was carried out using QIA Amp ® DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

PCR Amplification: Three oligonucleotide primer pairs for specific gene of *M. bovis* were used. The primers were prepared by Macrogen Company, South Korea.

PCR procedures for *M. bovis* (Perez-Casal and Prysliak, 2007): The PCR reaction mixture was 50µl. PCR was performed on a Bio-Rad thermal Cycler (S1000™ Thermal cycler, USA). Conditions for PCR were as follows: 1 cycle of 2 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec annealing at the appropriate temperature for the primer combination used (56°C *gap*A and p40 genes, 52°C *uvr*C gene) 2 min extension at 72°C and a final one cycle of 5 min at 72°C.

Sequencing and Sequence Analysis: The amplified fragments were purified using Gene Jet PCR purification kit; Fermentas (Cat no. KO701). Sequencing was performed by Macrogen Company (South Korea) and nucleotide and amino acid sequences of the *M. bovis* was compared with other strains published on GenBank using BLAST 2.0 search programs, (National Center for Biotechnology Information NCBI" http://www.ncbi.nlm.nih.gov/), respectively. The obtained nucleotide sequences comparisons and their various alignments with reference *M. bovis* were done using the Clustal software for multiple sequence alignment (Thompson *et al.*, 1994). The phylogentic trees were constructed using Clustal Omega for tree reconstruction of sequences by Neighbor-joining. Clustal Omega is accurate and allows alignments of any size to be analyzed (Sievers *et al.*, 2011).

RESULTS

At the Menofia Governate, the occurrence of *M. bovis* in nasal swabs, oral swabs and conjunctiva of cattle suffering from respiratory manifestation was 40, 25 and 20%, respectively.

PCR assays were used for identification of *M. bovis* isolates from calf cases: The examined *M. bovis* isolates yielded amplified fragments of respective size 1626 bp of *uvr*C gene, 1007 bp of *gap*A gene and 797 bp of p40 pseudogene (Fig. 1).

Sequence submission to GenBank: The following Acc. No. of *M. bovis* isolates from cattle at the Menofia Governorate of Egypt was KU559607, KU559606 and KU559608 for uvrC, GapA and P40 genes, respectively.

Nucleotide sequence analysis: Sequence analysis of *uvr*C gene nucleotide sequence of our field isolates showed (95.3%) similarity when compared with each other and (100%) identity with *M. bovis* reference strain (PG45) and the field strains on GenBank. Analysis of *gap*A gene nucleotide (nt.) sequence of *M. bovis* isolates from calves (Egy-8-Fa-14 and Egy-9-DK-14) showed (100%) identity between each other and (98.2%) identity with the reference strain (PG45). Our isolates showed (98.9% up to 100%) identity when compared with international field strains on GenBank. Concerning analysis of p40 pseudogene nucleotide sequence, our field isolates showed (97.5%) identity when compared with each other, while (Egy-12-Fa-14) showed (99.8%) similarity with *M. bovis* (PG45) reference strain and field strains on GenBank. The phylogenetic tree analyses were shown in Fig. 2-4. Fig. 2 demonstrates the phylogenetic analysis of 19 clones into 2 main clusters which was inferred from *uvr*C gene sequence data. Cluster 1 of *uvr*C gene represents sources of various clades originated from Egypt, Switzerland, China and Poland (14 clones). While the second cluster of *uvr*C gene was originated from Poland only (5 clones). Fig. 3 addresses 2 major clusters (of 9 clones) according to *gap* gene sequence data. Cluster 1 of *gap* gene represent similar clones from Egypt (2 different Provinces), China, while the other cluster match only reference strains of mycoplasma. Moreover, Fig. 4 discriminated 2 different clusters (of 12 clones) regarding p40 gene sequence data. The first cluster of p40 gene match sources from Egypt and China (9 clones), nevertheless, the 2nd cluster referred only to 3 reference Mycoplasma strains (3 clones).

Table 1: Primers used in PCR for detection of some genes in *M. bovis*

Primer	Sequence 5'-3'	Base pair	Reference
uvrC2-L	TTACGCAAGAGAATGCTTCA	1626	(Perez-Casal and Prysliak, 2007)
uvrC2-R	TAGGAAAGCACCCTATTGAT		
gap7	ATAGGAGGATCCAAAAGAGTCGCTATCAATGGTTTTGGACG	1007	
gap8	GGAAATGGTACCTTACTTAGTTAGTTTAGCAAAGTATGTTAATG		
P40-L	ATGAAAACAAATAGAAAAATAAGTC	797	(Thomas *et al.*, 2004)
P40-R	GTAGCTTTTTCCAATAATTTTCC		

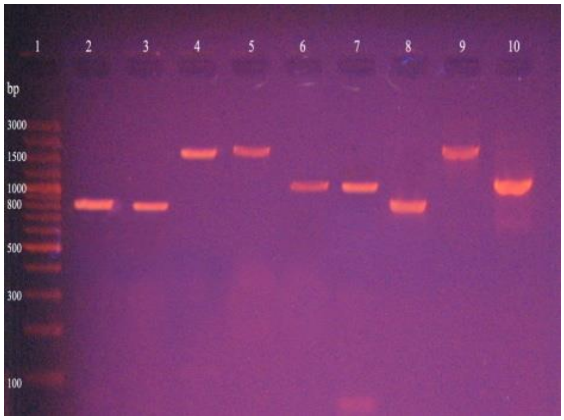

Fig. 1: *Mycoplasma bovis* confirmation using PCR. Lane 1, Gene Ruler 100 bp DNA Ladder (Fermentas) marker; lanes 2, 3, *M. bovis* (p40 gene); 4,5, *M. bovis* (*uvr*C gene); 6,7, *M. bovis* (*gap*A gene); 8, control positive *M. bovis* (p40 gene); 9, control positive *M. bovis* (*uvr*C gene); 10, control positive *M. bovis* (*gap*A gene).

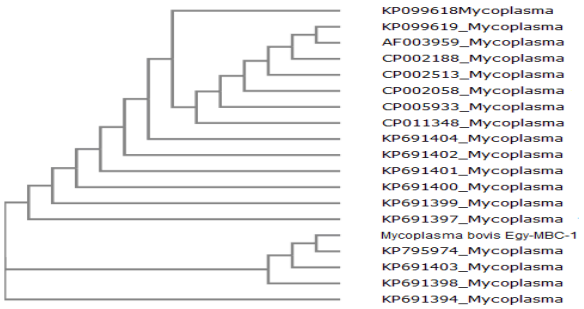

Fig. 2: The resulting relationships from Phylogenetic analysis for *M. bovis* which was inferred from *uvr*C gene sequence data.

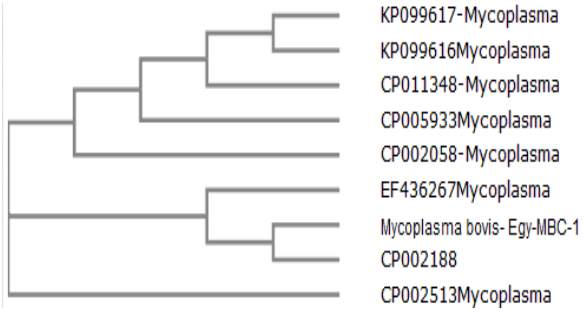

Fig. 3: The resulting relationships from phylogenetic analysis for *M. bovis* which was inferred from *gap*A gene sequence data.

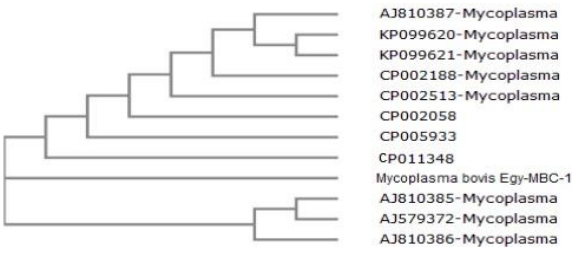

Fig. 4: The resulting relationships from phylogenetic analysis for *M. bovis* which was inferred from p40 gene sequence data.

DISCUSSION

M. bovis is estimated to be responsible for up to one third of the economic losses in the cattle industry caused by respiratory diseases (Nicholas and Ayling, 2003; Yilmaz *et al.*, 2016). The obtained result of nasal swabs (40%) was compared to previous findings in Poland (47.8%) (Szacawa *et al.*, 2015), the Czech Republic (8%) (Jozefová *et al.*, 2014), England and Wales (40.2%) (Aylinget *al.*, 2014**)** and France (55%) **(**Chazel *et al.*, 2010). Mycoplasmas were highly isolated from nasal swabs than from milk samples **(**Jozefová *et al.*, 2014**).** The difference of the current study findings with previous reports may be due to geographic distribution. Some mycoplasma may occur in several states with variant frequencies according to local epidemiological conditions **(**Baule *et al.*, 2001**)**. *M. bovis* has increasingly been recognized as one of the main pathogens involved in the bovine respiratory disease complex, alone or in association with other respiratory pathogens **(**Farshidet *al.*, 2002). Inter-strain whole genome comparison within *M. hyopneumoniae* was studied and provided evidence of intra species rearrangements resulting in strain-specific gene groups as well as evidences to factors related to pathogenicity (Vasconcelos *et al.*, 2005). A number of *M. agalactiae* surface ingredients has been shown to activate the host humoral response and includes lipoproteins such as p40 **(**Fleury *et al.*, 2002). *M. bovis* was identified previously by specific PCR for *M. bovis* and targeting the *uvr*C gene (Rossetti *et al.*, 2010). The PCR-based *uvr*C gene is more specific than Conventional PCR assays based the 16S rRNA gene (Pinnow *et al.*, 2001). The use of PCR primers targeting the 16S rRNA region is capable of differentiating many species of Mycoplasma, but may misdiagnose closely related species (Justice-Allen*et al.*, 2011). An example of this phenomenon is *M. bovis* and *M. agalactiae* which are identical in the 16S rRNA region. Characterization of closely related species occurs based upon other genes with known differences, such as the DNA repair gene, *uvr*C, which is used for *M. bovis* and *M. agalactiae* (Thomas *et al.*, 2004). As shown in Fig. 2, dendrogram for some of *M. bovis* was inferred from *uvr*C gene sequence data a strain from Poland as an out group. The dendrogram grouped 19 clones into 2 main clusters. The first cluster included strains from Egypt (No=2), China (No2), reference (No=4), Poland (No=6). The high degree of similarity was recorded between the Egyptian strain of Dakahlia and Swiss clone. The second cluster comprised 4 clones with a high degree of similarity between our isolate and a Polish clone. To understand the epidemiology of *M. bovis*, it is important to test the variation among *M. bovis* isolates. The genetic diversity in the current study was tested for some genes sequence using dendrogram analysis. Gene sequence was found to discriminate among the tested strains. Intra species, molecular difference was found among *M. bovis* strains from different countries. Cluster I was then grouped into variant subgroups. The pair wise variances among current Egyptian strain and other sub-lineages were estimated. The inter-lineage diversity was remarkable, indicating the nucleotide sequence variation with *uvr*C. Within cluster II, sub-groups had a closer relationship with *Mycoplasma bovis* Egy-MBC-1 and polish strains. Essential genes sequence analysis of genes other than the 16s rRNA genes may also be useful for classification of organisms (Olsen and Woese, 1993). Previously, two Hungarian *M. bovis* strains (MYC52) had identical sequence type as the reference clone PG45 (NCTC 10131) and were similar to a strain from Saudi Arabia (Sulyok *et al.*, 2014). Some Belgian and German *M. bovis* isolates had

the same sequence as PG45 (Manso-Silván *et al.*, 2012). Previous studies demonstrated that husbandry conditions influence the genetic diversity of *M. bovis*, with clones obtained from closed herds being less variable than those obtained from a multiple-source or open herds (Butler *et al.*, 2001). The examined Egyptian *M. bovis* population is genetically so similar. Potentially, the high genetic similarity of the Egyptian *M. bovis* slowness may be partially due to a single source of infection. Similar result investigated the diversity of sixty *M. bovis* strains in France before and after the year 2000 that clarified the homogenous pattern of the isolates after the year 2000 than older strains and this may indicate a single clone spread in the country in last years (Becker *et al*., 2015). Concerning the *gap* gene sequence analysis, the shown dendogram in Fig.3 reveals two major clusters. The first one included 5 clones originated from Egypt (No=2) and reference (No=3). The second cluster included our isolate from Menofia, Egypt, Canada; reference China (one each). The dendrogram out group clone was Chinese origin. Regarding phylogenetic tree of the nucleotide sequence of *M. bovis* p40 gene, our isolate was out grouped from 2 major clusters as shown in figure 4. Nevertheless, the first cluster included 2 Egyptian clones in previous studies, China and reference strains. The second cluster included 3 clones of India and Switzerland sources. The conservation of circulating *M. bovis* clones between Egypt and different countries may be due to the importation of cattle from Europe and India. On the other hand, (Butler *et al.*, 2001) reported a relation between source of infection as well as husbandry practice and the genetic profile of *M. bovis* isolates, so they expected multiple typing of *M. bovis* strains in open housed animals than in closed environment.

Conclusions: It could be concluded that *M. bovis* has a circulating and a conserved clone in Egypt. The dendrogram of some gene sequences of *M. bovis* recovered from Egypt revealed that there is similarity between the Egyptian and some international *M. bovis* strains. More studies are important required for investigation and molecular characterization of *M. bovis* strains especially in imported cattle to achieve effective control method for the disease.

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