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Identification and genetic characterization of Mycoplasma species affecting respiratory system in Egyptian cattle

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ABSTRACT

Mycoplasma species are the main important causative agents causing pneumonia in cattle. There are about 200 species or more. The current work aimed to investigate various *mycoplasma* spp. isolated from the respiratory tract of cattle by microbial culture, conventional polymerase chain reaction technique, and target gene sequencing with phylogenetic analysis. An initial screening was done to confirm the presence of *mycoplasma* spp. by culture on PPLO's agar, digitonin sensitivity and biochemical tests. 129 isolates were characterized by fried egg with depressed center colonies, digitonin sensitive and negative to glucose fermentation and arginine utilization test. Out of 305 samples, 20 samples were selected for amplification by PCR technique using *Mycoplasma* 16S rRNA primer. Seven samples were positive to *Mycoplasma* species and gave amplified band at 1013 bp. subsequently, the seven isolates were sequencing. Four sequenced isolates (EGS1, EGL6, EGL1 and EGS2) were closely related to each other and very close to *Mycoplasma bovis* strains and far from *M. leachii* 99/014/6. Two sequenced isolates (EGL2 and EGL5) were closely related to each other and very close to *Mycoplasma bovirhinis* strains and far from *M. leachii* 99/014/6. One sequenced isolate (EGL3) was very close to *Mycoplasma* arginini strain and far from *M. bovis* 99/014/6. From these results, we can conclude that conventional culture methods for diagnosis either by isolation and identification of *Mycoplasma* is a time-consuming method to diagnose *mycoplasma* infection. So, these methods can be replaced by PCR and genome analysis technology.

1. INTRODUCTION

Mycoplasma name is derived from the Greek mykes (fungus) and plasma (formed). In 1950, this name is used as an alternative to the term pleuropneumonia-like organisms (PPLO) (Alhaji *et al.*, 2020). *Mycoplasma* was firstly reported in Egypt by El-Ebeedy *et al*., (1985). The *Mycoplasma* infection was spread throughout the Egyptian farms and become endemic in some areas. Different species of *mycoplasma* were isolated from dairy Friesian cows and buffaloes with mastitis. *Mycoplasma* spp. included *M. bovis, M. bovigenitalium, M. dispar, M. bovirhinis and M. arginini. Mycoplasma bovis* are the main species, causes mastitis and arthritis in adults, but cause pneumonia, arthritis, and otitis media in calves (Al-Farha *et al.*, 2017).

Usually, the *mycoplasma* infection occurred without any clinical illness in the upper respiratory tract but once it reaches the lungs, it causes pneumonia. It differs from shipping fever pneumonia which is recognized in farms (Kashyap and Sarkar, 2010). *M. bovis* can transfer to the bloodstream and reach to joints, organs, and nerves. Commonly, it goes to the joints causing arthritis and tenosynovitis but can cause infection in the genitalia, udder, eyes, and ears (Hananeh *et al.*, 2018).

Diagnosis of *Mycoplasma* spp. is very difficult, and usually done by microbiological culture, because it is very simple and with low cost, but it has several disadvantages including that, the organisms must be shaded by the animal in viable form and still viable after collection till diagnosis in media to avoid false negative results, and its growth is very slow and the colonies may be still non- visible for several days (Parker *et al.*, 2018).

Due to *Mycoplasmas* specific structure, it cannot synthesize amino acids or fatty acids. So, the growth media must be specific and rich with peptone, serum, yeast extract and beef heart infusion with a final pH ranging from 7.3 to 7.8 (McVey et al., 2013). In another hand, recent PCR technique can be identified the DNA of the tested bacteria, with successful identification of *mycoplasma* spp. without amplification of *acholeplasma* spp. and minimized false positive results (Boonyayatra *et al.*, 2012).

There are different methods that allow genetic characterization of *mycoplasma* spp. Each method has points of strength and weakness, so a combination of them provides complete information, which allows the detection of the organism when correlated with clinical signs (Parker et al., 2018).

PCR involves cloning and sequencing of small DNA fragments which includes computational biology, making use of computational algorithms to assemble sequenced fragments. It is able to detect about 50 complete genomes of prokaryotes including *Mycoplasma*, which are currently

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available (*www.NCBI.nlm.nih.gov*). *Mycoplasma* genomes along with other families of prokaryotic genomes provide templates for comparative genomes (El-Metwally et al., 2013).

The gene sequence analysis of 16S rRNA is used for the identification and classification of prokaryotes as *mycoplasmas* spp. It can be amplified directly from the environment and used to determine the difference between microbes. The presence of difference between genomes, mosaicism, and lack of universal sequence limit 16S rRNA-based phylogenetic analysis. PCR-amplification bias and cloning bias can also result in an inaccurate representation of the microbial diversity (Rajendhran and Gunasekaran, 2011).

Sequence analysis of 16s rRNA is one of the main useful tools for phylogenetic analysis. In 1989, the first study of *mycoplasma* phylogeny occurred based on 16s rRNA sequences occurred by direct sequencing of rRNA with reverse transcriptase (Rajendhran and Gunasekaran, 2011).

This study was designed for the identification and isolation of different isolates of *Mycoplasma* spp. in cattle from four governorates in Egypt by PCR, followed by sequencing and phylogenetic analysis.

2. MATERIAL AND METHODS

2.1. Sample collection and animals:

A total of 305 samples were collected from cattle of different ages (more than two years for abattoir samples and between six months to two years for live animals) and sex as shown in Table (1) for bacteriological examination from different governorates in Egypt including Qalyubia, Giza, Menoufia, and Gharbia governorates. The samples were submitted for *Mycoplasma* isolation using conventional cultural technique followed by molecular identification of *Mycoplasma species* using PCR technique.

Table 1 Numbers and types of samples collected from cattle:

Animal status	Types of samples	Sites of samples collection	Animal condition		Total
			Apparently healthy	Diseased	
Living Cattle	Nasal Swabs	El-Qalyubia	8	7	15
		Giza	12	8	20
		Menoufia	25	9	34
		Gharbia	15	6	21
		Total	60	30	90
Slaughtered Cattle	TrachealSwabs	El-Bassatin	25	11	36
		Qalyub	15	9	24
		Total	40	20	60
	Lung Tissues	El-Bassatin	83	12	95
		Qalyub	51	9	60
		Total	134	21	155
Total Slaughtered			174	41	215
Total			234	71	305

2.2. Isolation of Mycoplasma species using the conventional cultural method:

It was performed according to Hazelton et al. (2018). *Mycoplasma* isolation from different samples was done by using *Mycoplasma* agar (HIMEDIA M266) and broth (HIMEDIA M268) supplemented with *Mycoplasma* enrichment supplement (HIMEDIA FD075).

It was inoculated into 5 ml *Mycoplasma* broth followed by incubation for 7 days at 37 ºC in a candle jar with elevated CO_2 levels, and examined for growth daily then sub-culturing is done into broth and plates. Plates were examined using a stereomicroscope to detect the characteristic fried egg colonies. Suspected samples were sub-cultured three times before being rejected as negative samples.

2.3. Biochemical identification of Mycoplasma isolates:

It was performed according to Freundt et al. (1973), Erno and Stepkovits (1973) by application of Digitonin sensitivity test, Glucose fermentation test and Arginine hydrolysis test.

2.4. Molecular identification of Mycoplasma isolates:

2.4.1. DNA extraction:

Using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH), DNA was extracted from twenty *Mycoplasma* isolates were chosen based on microscopical appearance after culture on PPLO agar media and biochemical identification with minor modifications using 100 µl of elution buffer not 200 µl to increase the concentration of DNA (Erfan and Shalaby, 2020). The sample suspension (200 µl) incubated with proteinase K (10 µl) and lysis buffer (200 µl) at 56 °C for 10 min. 200 µl of 100% ethanol was added after incubation, then washing and centrifugation of the sample. Elution of the nucleic acid with 100 µl of elution buffer.

2.4.2. Oligonucleotide Primer:

Primers used were supplied from Metabion (Germany) as shown in Table (2).

Table 2 Primers sequences, target genes and amplicon sizes of *Mycoplasma 16S rRNA*

Primers sequences	Amplified segment (bp)	Reference
GCTGGCTGTGTGCCTAATACA	1013 bp	Sayin, et al. (2016)
TGCACCATCTGTCACTCTGTTAACCTC		

2.4.3. PCR amplification:

Primers utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 5 µl of DNA template, 5.5 µl of water, and 1 µl of each primer of 20 pmol concentration. The reaction was performed in an Applied biosystem 2720 thermal cycler, as follows: Primary denaturation at 94 °C for 5 min, then 35 Amplification cycles (Secondary denaturation at 94 °C for 30 sec., Annealing at 56°C for 40 sec. and Annealing at 72 °C for one min., and Final extension at 72 °C for 10 min.

2.4.4. Analysis of the PCR Products:

Separation of PCR products was done by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, loading of 15 µl of the products in each gel slot. A gel pilot 100 bp plus ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and analysis of data through computer software.

2.4.5. Target gene sequencing and phylogenetic analysis:
Purification of PCR products was done by QIAquick PCR Product extraction kit (Qiagen, Valencia). Sequence reaction was done by Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) and then purification by Centrisep spin column. DNA sequences obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) was performed to establish sequence identity to GenBank accessions (Altschul et al., 1990). The phylogenetic tree created by the MegAlign module of LasergeneDNAStar version 12.1 Thompson et al. (1994) and Phylogenetic analyses was done using maximum likelihood, neighbor joining and maximum parsimony in MEGA6 (Tamura et al., 2013).

3. RESULTS

3.1. Microscopical examination of *Mycoplasma* isolates:
A total of 305 samples were cultivated on PPLO's agar. The microscopic examination revealed that 206 samples gave characteristic "fried egg colony" with depressed center colonies as shown in Figure (1).

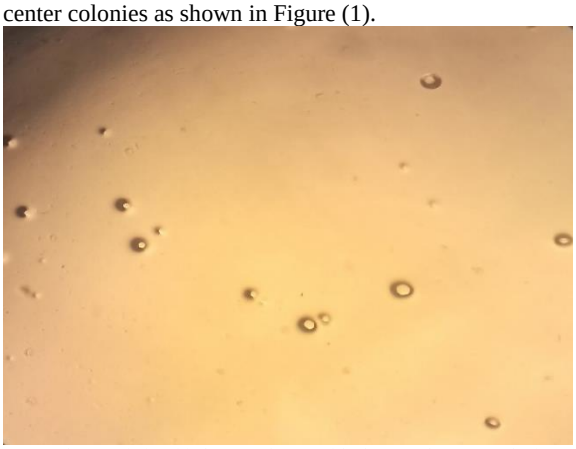

Fig. 1 characteristic "fried egg colony" with depressed center colonies of Mycoplasma isolates

3.2. Biochemical identification of *Mycoplasma* isolates:
A total of 173 *Mycoplasma* isolates were digitonin sensitive with > 5 mm zones of growth inhibition in digitonin disc diffusion assay. Furthermore, 129 isolates were negative for the glucose fermentation test and arginine hydrolysis test.

3.3. Identification of *Mycoplasma* isolates by using Polymerase Chain Reaction (PCR):
Out of the 20 *Mycoplasma* isolates, seven were identified as *Mycoplasma* species by PCR targeting *Mycoplasma* 16S rRNA gene representing 35 % of *Mycoplasma* isolates (Figure 2 and 3).

3.4. Gene targeted sequencing result:
Sequencing of *Mycoplasma* 16S rRNA gene was conducted in both directions and a consensus sequence of 1013 bp was used for nucleotides analysis. Seven isolates were submitted to the GenBank database with the following accession numbers: MW496836 (*M. bovis* EGS1), MW493232 (*M. bovis* EGL6), MW493231 (*M. bovis* EGL1), MW496837 (*M. bovis* EGS2), MW493226 (M. arginini EGL3), MW493227 (*M. bovirhinis* EGL2) and MW496423 (*M. bovirhinis*EGL5).

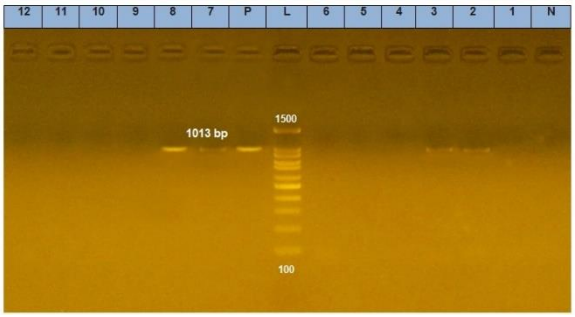

Fig. 2 Shows agarose gel electrophoresis with amplification of 1013 bp fragment of *Mycoplasma* 16S rRNA. Lane (L): DNA ladder, Lane (P): Reference strain (control positive), Lane (N): Negative control, Lane (2,3,7 and 8): *Mycoplasma* positive (samples), Lane (1,4,5,6,9,10,11 and 12): *Mycoplasma* negative (samples).

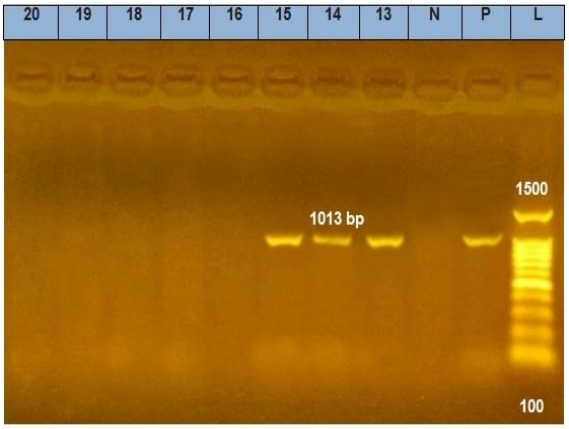

Fig. 3 Shows agarose gel electrophoresis with amplification of 1013 bp fragment of *Mycoplasma* 16S rRNA Lane (L): DNA ladder, Lane (P): Reference strain (control positive), Lane (N): Negative control, Lane (13,14 and 15): *Mycoplasma* positive (samples), Lane (16- 20): *Mycoplasma* negative (samples)

3.5. Sequence distance of the examined samples:
Four sequenced isolates (EGS1, EGL6, EGL1 and EGS2) were related closely to each other and very close to *Mycoplasma bovis* strains and far from *M. leachii* 99/014/6. Two sequenced isolates (EGL2 and EGL5) were related closely to each other and very close to *Mycoplasma bovirhinis* strains and far from *M. leachii* 99/014/6. One sequenced isolate (EGL3) was very close to *Mycoplasma* arginini strain and far from *M. bovis* 99/014/6, as shown in Figure (4).

3.6. Phylogenetic analysis of the samples
The seven sequenced isolates in this study are distinct from other field isolates from Egypt and other countries as shown in Figure (5). They were placed in three groups according to similarity percent. The tree indicates that four sequenced isolates (EGS1, EGL6, EGL1 and EGS2) were closely related to each other (99.8-100% identity) and very close to *Mycoplasma bovis* strains (99.6-100% identity) and far from *M. leachii* 99/014/6 (79.5% identity). Two sequenced isolates (EGL2 and EGL5) were closely related to each other (100% identity) and very close to *Mycoplasma bovirhinis* strains (99.6-100% identity) and far from *M. leachii* 99/014/6 (79% identity). One sequenced isolate (EGL3) was very close to *Mycoplasma* arginini strain (99.8-100% identity) and far from *M. bovis* 99/014/6 (3.6-83.9% identity).

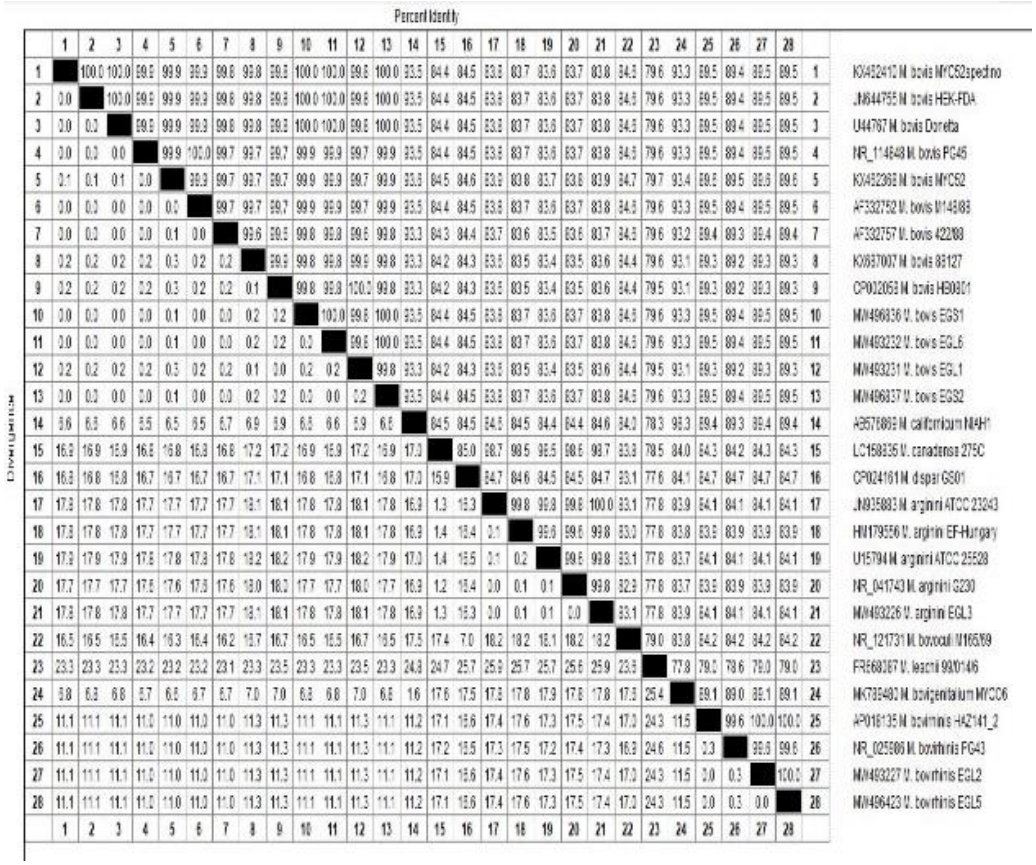

Fig. 4 Sequence distance of the examined samples

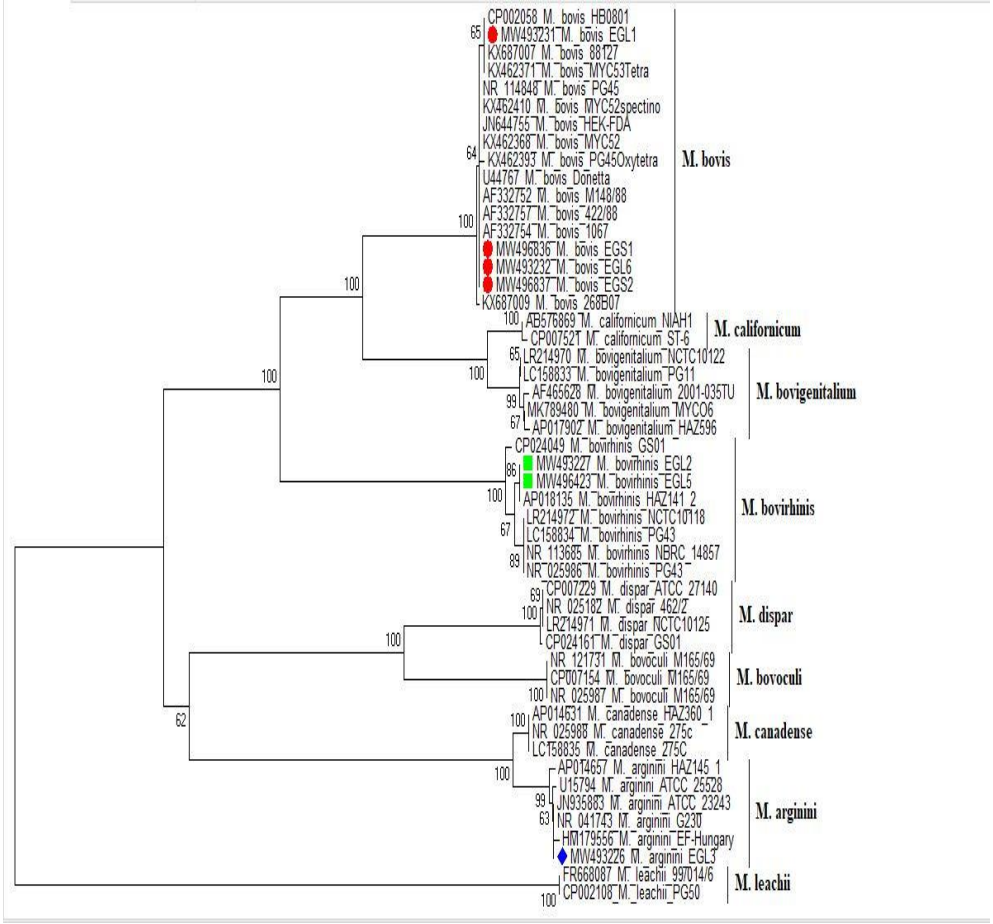

Fig. 5 Phylogenetic analysis of the samples

4. DISCUSSION

The present research aimed to study a molecular characterization of *Mycoplasma spp*. affecting the respiratory system of cattle in Egypt. A total of 305 samples revealed that 206 samples appeared with a characteristic fried egg with a percent of 68 %. One hundred and seventy-three isolates were digitonin positive (Family *Mycoplasmataceae*) in a percentage of 84 %. This incidence is higher than that reported by Metwalli (1980) (50 %) but, lower than that reported by Boonyayatra et al., (2012) (92%).

In this study, twenty *Mycoplasma* isolates were chosen based on microscopical appearance and biochemical identification were characterized by PCR by using *Mycoplasma* 16S rRNA primer at 1013 bp against *Mycoplasma* spp. (Sayin et al., 2016). The positive results of PCR were seven samples with a percent of 35 %. These results were lower than the culture result (68%). This is due to the high sensitivity and specificity of PCR (OIE, 2008).

PCR is used to diagnose *Mycoplasma* species from different samples with high specificity, sensitivity, and efficiency for laboratory diagnosis in comparison with other conventional culture methods (Waites et al., 2012).

PCR is used to detect *M. bovis* in the 1990s, by targeting 16S rRNA gene, which is the most important gene targeted for bacterial diagnosis because it is present in all types of bacteria with unchanged function. The results of PCR do not take a long time, it appears within a day so, the PCR method is more rapid than culture (Parker et al., 2018).

Although PCR technique has several advantages, it also has some disadvantages as serious contamination problems due to improper handling of the DNA tested sample inducing false results (Levisohn and Kleven, 2000).

Mycoplasma's genome analysis of bovine origin characterized by a difference in pathogenicity, host and tissue tropism, will increase the information about bovine *mycoplasmas* evolution and will improve the ability to resolve the pathogenicity or host specificity of the genetic basis of *mycoplasma* (Manso-Silván, et al., 2013).

In the current study, the *Mycoplasma* 16S rRNA gene was the target gene for the DNA sequencing as *Mycoplasma* 16S rRNA was characterized by the presence of nucleotide insertion and deletion, which may be helpful for differentiation of strains (Sayin et al., 2016).

Three sequenced isolates *M. bovis* EGS1 (Accession no. MW496836), *M. bovis* EGL6 (Accession no. MW493232) and *M. bovis* EGS2 (Accession no. MW496837) showed 100% maximum identity to 12 *M. bovis* strains. Including *M. bovis* MYC52 spectino strain (Accession no. KX462410), which shows high antimicrobial sensitivity to fluoroquinolones group of antibiotics (Sulyok et al., 2014).

One sequenced isolate *M. bovis* EGL1 (Accession no. MW493231) showed 100% maximum identity to 15 *M. bovis* strains, including the *M. bovis* HB0801 strain (Accession no. CP002058), which used to develop a live vaccine for the prevention of *M. bovis* in cattle by *M. bovis*-150 the attenuated strain after 150 *in vitro* passages (Khan et al., 2017).

Two sequenced isolates *M. bovirhinis* EGL2 (Accession no. MW493227) and *M. bovirhinis* EGL5 (Accession no. MW496423) showed 100% maximum identity to 10 *M. bovirhinis* strains including the *M. bovirhinis* HAZ141_2 strain (Accession no. AP018135), which the complete genome sequence was isolated from bovine nasal discharge in Japan (Hata et al., 2017), The evolutionary relationships and virulence factors of *M. bovirhinis* are still poorly understood (Chen et al., 2018).

One sequenced isolate *M. arginini* EGL3 (Accession no. MW493226) showed 100% maximum identity to 15 strains of *M. arginine* including the *Mycoplasma arginini* Strain HAZ 145_1 (Accession no. AP014657), which the complete genome sequence was isolated from bovine specimens in Japan, *M. arginini* has been isolated from various mammalian host, and may become a precipitating factor for mastitis by other bacteria, and has unspecific pathogenicity against cows (Hata, 2015).

5. CONCULOSIONS

Mycoplasmas cause some of the most serious and economically most costly diseases of cattle, and diagnosis by conventional culture methods is time-consuming so, these methods can be replaced by PCR technique which has several advantages, but it also has some disadvantages as cost and serious contamination problem due to improper handling of the DNA tested sample inducing false results. And Comparative genome analysis of *Mycoplasmas* will improve our understanding of the evolution of bovine *Mycoplasmas* and will clarify the genetic basis of *mycoplasma* pathogenicity and host specificity.

6. REFERENCES

1. Al-Farha, A.A., Hemmatzadeh, F., Khazandi, M., Hoare, A., Petrovski, K. (2017). Evaluation of effects of Mycoplasma mastitis on milk composition in dairy cattle from South Australia. BMC Veterinary Research 13, 351-359.
2. Alhaji, N.B., Ankeli, P.I., Ikpa, L.T., Babalobi, O.O. (2020). Contagious Bovine Pleuropneumonia, challenges and prospects regarding diagnosis and control strategies in Africa. Veterinary Medicine (Auckland, N.Z.) 11, 71-85.
3. Boonyayatra, S., Fox, L.K., Gay, J.M., Sawant, A., Besser, T.E. (2012). Discrimination between *Mycoplasma* and *Acholeplasma* species of bovine origin using digitonin disc diffusion assay, nisin disc diffusion assay, and conventional polymerase chain reaction. Journal of Veterinary Diagnostic Investigation 24(1), 7-13.
4. Chen, S., Hao, H., Zhao, P., Liu, Y., Chu, Y. (2018). Genome-wide analysis of *Mycoplasma bovirhinis* GS01 reveals potential virulence factors and phylogenetic relationships. G3: Genes, Genomes, Genetics 8(5), 1417–1424.
5. El-Ebeedy, A.A., Gad, A.S., Rashwan, A., Mostafa, A., El-Ahl, S.S., Esmail, S., Allam, N.M. (1985). Isolation of *Mycoplasma bovis* from an outbreak of bovine mastitis in Egypt. Journal of the Egyptian Veterinary Medical Association 45(1), 247 - 253.
6. El-Metwally, S., Hamza, T., Zakaria, M., Helmy, M. (2013). Next-generation sequence assembly, four stages of data processing and computational challenges. PLoS Computational Biology 9(12), e1003345.
7. Erfan, A.M., Shalaby, A.G. (2020). Genotyping of rabbit hemorrhagic disease virus detected in diseased rabbits in Egyptian Provinces by *VP60* sequencing. Veterinary World 13(6), 1098-1107.
8. Erno, H., Stipkovits, L. (1973). Bovine *Mycoplasmas*, cultural and biochemical studies. Acta Veterinaria Scandinavica 14(3), 450- 463.
9. Freundt, E. (1973). Type strains of species of the order *Mycoplasma tales*, including designation of neotypes for *Mycoplasma mycoides subsp. mycoides*, *Mycoplasma agalactiae subsp. agalactiae*, and *Mycoplasma arthritidis*. International Journal of Systematic and Evolutionary Microbiology 23(1), 55-61.
10. Hananeh, W.M., Al Momani, W.M., Ababneh, M.M., Abutarbush, S.M. (2018). *Mycoplasma bovis* arthritis and pneumonia in calves in Jordan, An emerging disease. Veterinary World 11(12), 1663-1668.
11. Hata, E. (2015). Complete genome sequence of *Mycoplasma arginine* strain HAZ 145_1 from bovine mastitic milk in Japan. Genome announcements 3(2), e00265-15.

12. Hata, E., Nagai, K., Murakami, K. (2017). Complete genome sequence of *Mycoplasma bovirhinis* strain HAZ141_2 from bovine nasal discharge in Japan. Genome announcements 5(39), e01000-17.
13. Hazelton, M.S., Sheehy, P.A., Bosward, K.L., Parker, A.M., Morton, J.M., Dwyer, C.J., Niven, P.G., House, J.K. (2018). Short communication, shedding of *Mycoplasma bovis* and antibody responses in cows recently diagnosed with clinical infection. Journal of Dairy Science 101(1), 584–589.
14. Kashyap, S., Sarkar, M. (2010). *Mycoplasma pneumonia*, Clinical features and management. Lung India, Official Organ of Indian Chest Society 27(2), 75–85.
15. Khan, F.A., Zhao, G., Guo, Y., Faisal, M., Chao, J., Chen, X., He, C., Menghwar, H., Dad, R., Zubair, M., Hu, C., Chen, Y., Chen, H., Rui, Z., Guo, A. (2018). Proteomics identification and characterization of MbovP730 as a potential DIVA antigen of *Mycoplasma bovis*. Oncotarget 9(47), 28322–28336.
16. Levisohn, S., Kleven, S.H. (2000). Avian mycoplasmosis (Mycoplasma gallisepticum). Revue scientifique et technique 19(2),425-442.
17. Manso-Silván, L., Tardy, F., Baranowski, E., Barré, A., Blanchard, A., Breton, M., Couture, C., Citti, C., Dordet-Frisoni, E., Dupuy, V., et al. (2013). Draft genome sequences of *Mycoplasma alkalescens*, *Mycoplasma arginini*, and *Mycoplasma bovigenitalium*, three species with equivocal pathogenic status for cattle. Genome announcements 1(3), e00348-13.
18. McVey, D.S., Kennedy, M., Chengappa, M.M. (2013). Veterinary Microbiology. Hoboken, Wiley.
19. Metwalli, A.S. (1980). Studies on mycoplasma in poultry. M. V. Sc. Thesis. Faculty of Veterinary Medicine Cairo University.
20. OIE, (2008). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Sixth Edition. Office international des epizooties, Paris, France.
21. Parker, A.M., Sheehy, P.A., Hazelton, M.S., Bosward, K.L., House, J.K. (2018). A review of mycoplasma diagnostics in cattle. Journal of Veterinary Internal Medicine 32, 1241–1252.
22. Rajendhran, J., Gunasekaran, P. (2011). Microbial phylogeny and diversity, Small subunit ribosomal RNA sequence analysis and beyond. Microbiological Research 166(2), 99–110.
23. Sayin, Z., Sakmanoğlu, A., Uçan, US., Uslu, A., Hadimli, H.H., Aras, Z., Özdemir, O., Erganiş, O. (2016). *Mycoplasma* infections in dairy cattle farms in Turkey. Turkish Journal of Veterinary and Animal Sciences 40, 569-574.
24. Sulyok, K.M., Kreizinger, Z., Fekete, L., Hrivnák, V., Magyar, T., Jánosi, S., Schweitzer, N., Turcsányi, I., Makrai, L., Erdélyi, K., et al. (2014). Antibiotic susceptibility profiles of *Mycoplasma bovis* strains isolated from cattle in Hungary, Central Europe. BMC Veterinary Research 10(1), 256–264.
25. Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013). MEGA6, molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30, 2725–2729.
26. Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994). Clustal W, improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap pentalties and weight matrix choice. Nucleic Acids Research 22(22), 4673-4680.
27. Waites, K.B., Xiao, L., Paralanov, V., Viscardi, R.M., Glass, J.I. (2012). Molecular methods for the detection of Mycoplasma and ureaplasma infections in humans, a paper from the 2011 William Beaumont Hospital Symposium on molecular pathology. The Journal of Molecular Diagnostics 14(5), 437–450.