Isolation and Molecular Characterization of *Mycoplasma arginini* isolated from sheep and goats with respiratory manifestation in Dakahilia Governorate

Elshafay, Dina, Y.[*]; Nermin Awad[**]; Yasser F. Elnaker.[***] and Emad E. Younis [****]

[*]Mycoplasma Department, Animal Health Research Institute, Dokii, Giza.
[**]Fellow of Bacteriology, mycology and immunology, Faculty of veterinary Medicine, Mansoura University
[***]Department of Animal medicine, Fac. Vet. Med. New valley, Assiut University.
[****]Department of Internal medicine and infectious diseases, Fac. Vet. Med, Mansoura Univ.

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Abstract

In this study a total of 160 Nasal swabs were collected from sheep (79) and goats (21) suffer from respiratory manifestation, *Mycoplasma spp.* was isolated from 40 samples (25%) from both sheep and goats. In sheep the percent of isolation was (25.32%) 20 isolate out of 79 nasal swabs, while the percent of isolation in goat was (24.69%) 20 isolate out of 81 nasal swabs examined. According to age the higher percent of isolation was found in age group (1- 2) year old sheep (50%) as 7 isolates out of 14 nasal swabs examined, while in goat the higher percent of isolation was in age group (6-12 month old) by 47.83% (11 isolates out of 23 nasal swabs examined. The lower isolation rates were found in adult sheep and goat (> 2 year old) by 15.38% in sheep and 24.69% in goat. Mycoplasma isolates was molecularly characterized by PCR and DNA sequencing as *Mycoplasma arginini* and submitted to Gen Bank. An accession number has been assigned to each nucleotide sequence and was KP972458-KP972459.

Key words: *Goat – Mycoplasma - PCR – sheep pneumonia .*

Introduction

Respiratory disease in sheep and goats can result in sudden death or in protracted illness. Pneumonia may occur in all ages of sheep, its severity is directly related to reduced growth and feed efficiency. In many sheep flocks pneumonia is a major cause of lamb mortality **(Thonney *et al.*, 2008),** Pneumonia in small ruminants constitutes a serious setback to the growth of this group of animals with resultant economic losses due to the risk of death with the contagious caprine pleuropneumonia (CCPP) **(Ruragirwa and McGuire, 2003).** The infecting agent could be one of bacteria, chlamydia, mycoplasma, or viruses. The most common cause of pneumonia, particularly in sheep, is pasteurellosis or mannheimiosis, while mycoplasmal pneumonia is often overlooked.

Other bacteria occasionally associated with respiratory disease in small ruminants are *Arcanobacterium pyogenes, Staphylococcus spp., Streptococcus spp., Haemophilus spp., and Klebsiella pneumonia* **(Nicholas *et al.*, 2008a).** Even though *Mycoplasma arginini* is an important pathogen by itself in sheep and goats, co infection by *M. haemolytica* intensifies the pathologic injury of pneumonia (**Lin *et al.*, 2008; *(*Nicholas *et al.*, 2008b)**. *Mycoplasma arginini* has been also isolated from cases of ovine keratoconjunctivitis. **Leach (1970)** and **Cottew (1979)** reported this organism's fre-

quent occurrence in pneumonic sheep lungs, mouth, and esophagus, a finding that has been confirmed (A. J. DaMassa, unpublished data) **Ayşe Kılıc *et al.*,** (**2013)**.

Mycoplasma arginini is a mammalian parasite found in a number of animal species.

Accumulating evidence in the case reports suggests the pathogenic role of *M. arginini* as a new human zoonosis. **Yechouron *et al.*, (1992).** Thus our work is to investigate more about the role of *M. arginini* in the respiratory affections in sheep and goat beside the phylogenic analysis of native strains.

Materials and Methods

Animals

A total of 160 sheep and goats from six flocks in Dakahillia Governorate suffering from respiratory manifestations were examined clinically and, then samples were collected for mycoplasma isolation.

Samples

One hundred and sixty nasal swabs were collected from sheep and goats suffering from respiratory manifestation (**Table 1),** the swabs were preserved at – 20°c till used.

Table (1). Nasal swabs collected from sheep and goats showing respiratory manifestation from different localities in Dakahlia Governorates.

Flock	Total number	Sheep	Goat
No. 1	30	19	11
No. 2	20	10	10
No. 3	30	10	20
No. 4	20	10	10
No. 5	40	20	20
No. 6	20	10	10
Total	**160**	**79**	**81**

Isolation of Mycoplasma:

Media used for cultivation and isolation of Mycoplasma:

Liquid and solid media for the isolation and propagation of mycoplasma were prepared as described by **Sabry and Ahmed (1975).**

Digitonin sensitivity test was done for the obtained isolates according to **Erno and Stipkovits (1973).**

Biochemical characterization was carried out by glucose fermentation and arginine deamination tests as described by **Erno and Stipkovits (1973)**. Film and spot formation medium **(Fabricant and Freundt, 1967).**

Polymerase chain reaction (PCR):

Preparation of samples for DNA extraction (**Yleana *et al.*, 1995)** B-Primer selection.

Table (2). Primers of mycoplasma

Mycoplasma Species	Forwarded primer	Reverse primer	product size (bp)	Thermal cycle	Reference
Sequence of 16S common gene for ruminant Mycoplasma	MunivF 5- AGA TC CTA CGG GAG CA GCA -3	MunivR 5' ACT GC GAT TCC GAC TTC ATG 3'	1000	Initial denaturation step at 94°C for 5 min., followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min., and extension at 72°C for 1.5 min. A final extention step at 72°C for 10 min	**Alberto *et al* (2006)**
Mycoplasma mycoides Subspecies *capri*	5-ACTGAG-CAATTCCTCTT-3	5-TTAATAAGTCTC TATATGAAT-3	195	Initial denaturation 94ºC for 5 min, Then 35 cycles of Denaturation 94ºC for 1min, Annealing 46ºC for 90sec, Extension 72ºC for 1min, final Extension 94ºC for 10 min.	**Vijay *et al* (2013)**
Mycoplasma agalactiae	MAGAUVRC1-L 5' - CTC AAA AAT ACA TCA ACA AGC - 3'	MAGAUVRC1-R 5' - CTT CAA CTG ATG CAT CAT AA - 3'	1624	initial sample denaturation, 5 min at 95°C 35 cycles: denaturation, 30 s at 94°C; annealing, 30 s at 50° C; extension, 1 min at 72°C - completion of the amplification process by final extension at 72°C for 10 min	**ZENDULKOVA *et al* (2007)**
Mycoplasma ovipneumoniae (16S–23S intergenic spacer)	MoIGSF GGAACACCTCCT TTCTACGG	MoIGSR CCAAGGCATC-CACCAAATAC	390	initial denaturation step at 95°C (15 min) 30 cycles at 95°C for 30 s, at 58°C for 30 s, and at 72°C for 30 fi nal extension step at 72°C (5 min	**Thomas *et al* (2012)**

PCR amplification for Mycoplasma was performed in 50 µl reaction mixture consisting of 5 µl of 50 ng Mycoplasma genomic DNA, 25 µl of 2 x Master mix (Multiplex gen) VIVANTIS, 1 µl of 50 pmol of each primer, 0.5 mM MgCl, and 35µl of DNase- RNase- free, deionized water. DNA amplification was performed as shown in Table (2), Following amplification, 5 µl of each amplicon was mixed with sample buffer and applied on agarose gel 1% (w/v) containing 0.5 µg of ethidium bromide. The samples were electrophoreses at 50 volts for 20 min on a horizontal electrophoresis unit. A 100 bp DNA ladder was used as molecular weight standard (VIVANTIS). After electrophoresis, the gel was visualized photographed.

Selected published sequences of 16S rRNA genes **which were used in sequence analysis and phylogeny**:-

<M.arginini.ATCC23243 gb|JN935883.1|:319-1256

<M.arginini.D7 gb|HQ661822.1|:269-1206

<M.arginini.E3.5 gb|HQ661820.1|:269-1206
<M.arginini.787 gb|HQ661826.1|:269-1207
<M.arginini.HAZ145_1
dbj|AP014657.1|:140970-141907
<M.arginini.C2-Ass-11 gb|JN543264.1|:1-932
<M.arginini.E2.5 gb|HQ661827.1|:350-1287
<M.arginini.C1-Beh-10 gb|HM635904.1|:1-932
<M.phocicerebrale.1049 gb|JN935885.1|:320-1257
<M.auris.UIA ref|NR_026035.1|
<M.neophronis.G.A ref|NR_108494.1|
<M.cloacale.383 ref|NR_024985.1|:308-1244
<M.salivarium.PG20 ref|NR_041745.1|:310-1247
<M.hyosynoviae.S-16 ref|NR_029183.1|:341-1277

Sequence and phylogenetic analysis:-

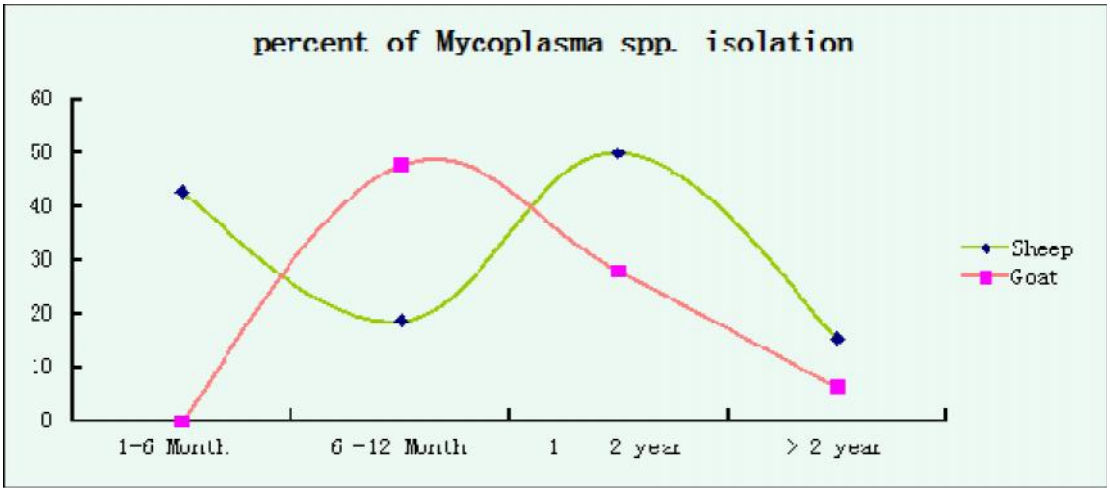
Sequencing of the PCR products of 16S rRNA gene were conducted in both directions and a consensus sequence of 943 bp was used for nucleotide analysis. The original sequence was trimmed to remove ambiguous nucleotide sequences usually exist in the beginning of the sequencing reaction. Identification of homologies between nucleotide sequences of the *M. arginini* were compared with other strains published on Gen Bank using BLAST 2.1 and PSI-BLAST search programs, (National Center for Biotechnology Information "NCBI" http:// www.ncbi.nlm.nih.gov/). The obtained nucleotide sequences comparisons and their multiple alignments with reference M. arginini and other mycoplasmas sequences were done using the BioEdit sequence alignment editor, (**Hall, 1999)** and MegAlign™ (DNASTAR, Lasergene®, Version 7.1.0, USA). 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 (**Thompson *et al., 1994)***. Clustal V was used when end gaps were faced. Sequence divergence and identity percent's were calculated by MegAlign™. The obtained sequences were submitted to Gen Bank (after trimming and correction) and retrieved accession numbers; KP972458 and KP972459 for samples sequences which designated Dak-1/M.arg/EG014 and Dak-2/M.arg/EG014 respectively

Results

Table (3). Percent of *Mycoplasma spp.* isolation from sheep and goats, with respiratory manifestation according to age.

Species / Age	Sheep			Goat			Total		
	Total	+ve	%	Total	+ve	%	Total	+ve	%
1-6 Month	7	3	42.86	2	0	0	9	3	33.33
6 -12 Month	32	6	18.75	23	11	47.83	53	17	32.08
1 – 2 year	14	7	50	25	7	28	39	14	35.9
> 2 year	26	4	15.38	31	2	6.45	59	6	10.17
Total	**79**	**20**	**25.32**	**81**	**20**	**24.69**	**160**	**40**	**25**

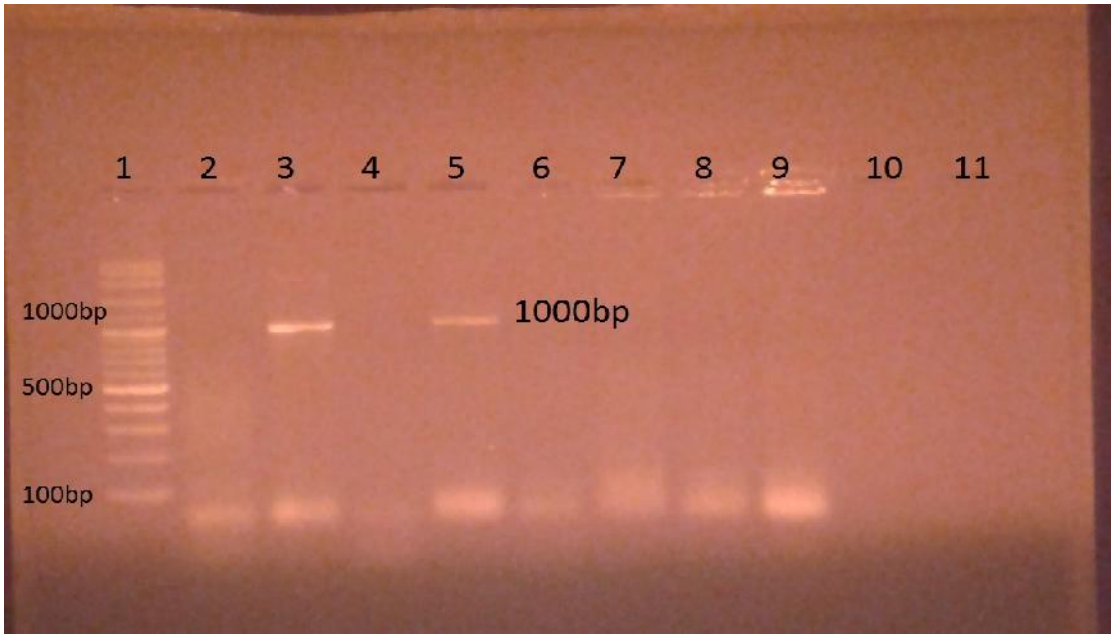
Fig (1). Percent of *Mycoplasma spp.* isolation from sheep and goats, with respiratory manifestation according to age.



These results are summarized in Table (3) and Fig (1) explained that *Mycoplasma spp.* Was isolated in 40 (25%) nasal swab samples out of 160 nasal swabs collected from sheep and goats with respiratory manifestation., in sheep the percent of isolation were (25.32%) 20 isolate out of 79 nasal swab while the percent of isolation in goat were (24.69%) 20 isolate out of 81 nasal swab examined.

According to age the higher percent of isolation were found in age group (1- 2) year old sheep (50%) as 7 isolates out of 14 nasal swab examined, while in goat the the higher percent of isolation were in age group (6-12 month old) by 47.83% (11 isolates out of 23 nasal swab examined. The lower isolation rate were found in adult sheep and goat (> 2 year old) by 15.38% in sheep and 24.69% in goat.

Fig (2): Gel electrophoresis of PCR products of mycoplasma 16s rRNA gene. 1: VC100BP Puls DNA Ladder , Lane 3and 5: The amplified products prepared from positive Nasal Swabs of diseased sheep, Lane 2: negative control ,Lanes 4 &6-11 negative samples .



PCR results

Of the 40 cases found positive with genus-specific primers, 20 isolate from sheep and 20 isolates from goat were positive with species-specific primers for *Mycoplasma*. The bands obtained were 1000 bp (**Fig. 2**).

The isolates in this study were classified into two different groups according to their phylogenetic relatedness. There were no differences in the nucleotide sequences of the 16s RNA gene among members of these two different groups.

Phylogenetic distances among the strains are shown in Table (4) Figure (3). Among the isolates, two showed identical nucleotide sequence, meaning they represent the same strains. Isolates KP972458 and KP972459 for samples sequences which designated Dak-1/ M.arg/EG014 and Dak-2/M.arg/EG014 respectively

Table (4). The percentages of identities and diversities of nucleotide sequences of the *Mycoplasma arginini* 16s rRNA in the present study.

Sequence pair distances of Mycoplasma arginini 16S rRNA gene
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Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
1		98.6	99.4	99.4	99.4	99.4	99.1	99.4	98.9	99.0	98.3	98.1	96.8	96.7	96.4	96.2	1	Dak_1-M.arg-EG014
2	1.4		98.9	98.9	98.9	98.9	98.7	98.9	98.5	98.9	97.8	97.6	96.3	96.2	95.9	95.7	2	Dak_2-M.arg-EG014
3	0.6	1.1		100.0	100.0	100.0	99.8	100.0	99.6	99.5	98.9	98.7	97.4	97.3	97.0	96.8	3	M.arginini.ATCC23243
4	0.6	1.1	0.0		100.0	100.0	99.8	100.0	99.6	99.5	98.9	98.7	97.4	97.3	97.0	96.8	4	M.arginini.D7
5	0.6	1.1	0.0	0.0		100.0	99.8	100.0	99.6	99.5	98.9	98.7	97.4	97.3	97.0	96.8	5	M.arginini.E3.5
6	0.6	1.1	0.0	0.0	0.0		99.8	100.0	99.6	99.5	98.9	98.7	97.4	97.3	97.0	96.8	6	M.arginini.787
7	0.9	1.3	0.2	0.2	0.2	0.2		99.8	99.4	99.2	98.7	98.5	97.2	97.1	96.8	96.7	7	M.arginini.HAZ145_1
8	0.6	1.1	0.0	0.0	0.0	0.0	0.2		99.6	99.5	98.9	98.7	97.4	97.3	97.0	96.8	8	M.arginini.C2-Ass-11
9	1.1	1.5	0.4	0.4	0.4	0.4	0.6	0.4		99.0	98.5	98.3	97.0	96.9	96.6	96.4	9	M.arginini.E2.5
10	1.0	1.1	0.5	0.5	0.5	0.5	0.8	0.5	1.0		98.4	98.2	96.9	96.8	96.7	96.5	10	M.arginini.C1-Beh-10
11	1.7	2.2	1.1	1.1	1.1	1.1	1.3	1.1	1.5	1.6		98.7	97.7	97.3	96.8	96.7	11	M.phocicerebrale.1049
12	1.9	2.4	1.3	1.3	1.3	1.3	1.5	1.3	1.7	1.9	1.3		97.2	97.2	96.9	96.8	12	M.auris.UIA
13	3.3	3.8	2.6	2.6	2.6	2.6	2.8	2.6	3.1	3.2	2.4	2.8		97.3	96.2	96.4	13	M.neophronis.G.A
14	3.4	3.9	2.7	2.7	2.7	2.7	2.9	2.7	3.2	3.3	2.7	2.8	2.7		96.1	95.9	14	M.cloacale.383
15	3.7	4.3	3.1	3.1	3.1	3.1	3.3	3.1	3.5	3.4	3.3	3.2	4.0	4.1		97.5	15	M.salivarium.PG20
16	4.0	4.5	3.3	3.3	3.3	3.3	3.4	3.3	3.7	3.6	3.4	3.3	3.7	4.2	2.5		16	M.hyosynoviae.S-16
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		

Fig (3). Phylogenetic tree of *Mycoplasma arginini* 16s rRNA gene from sheep and goat with other mycoplasma that were taken from the Gene Bank database 16s rRNA gene.

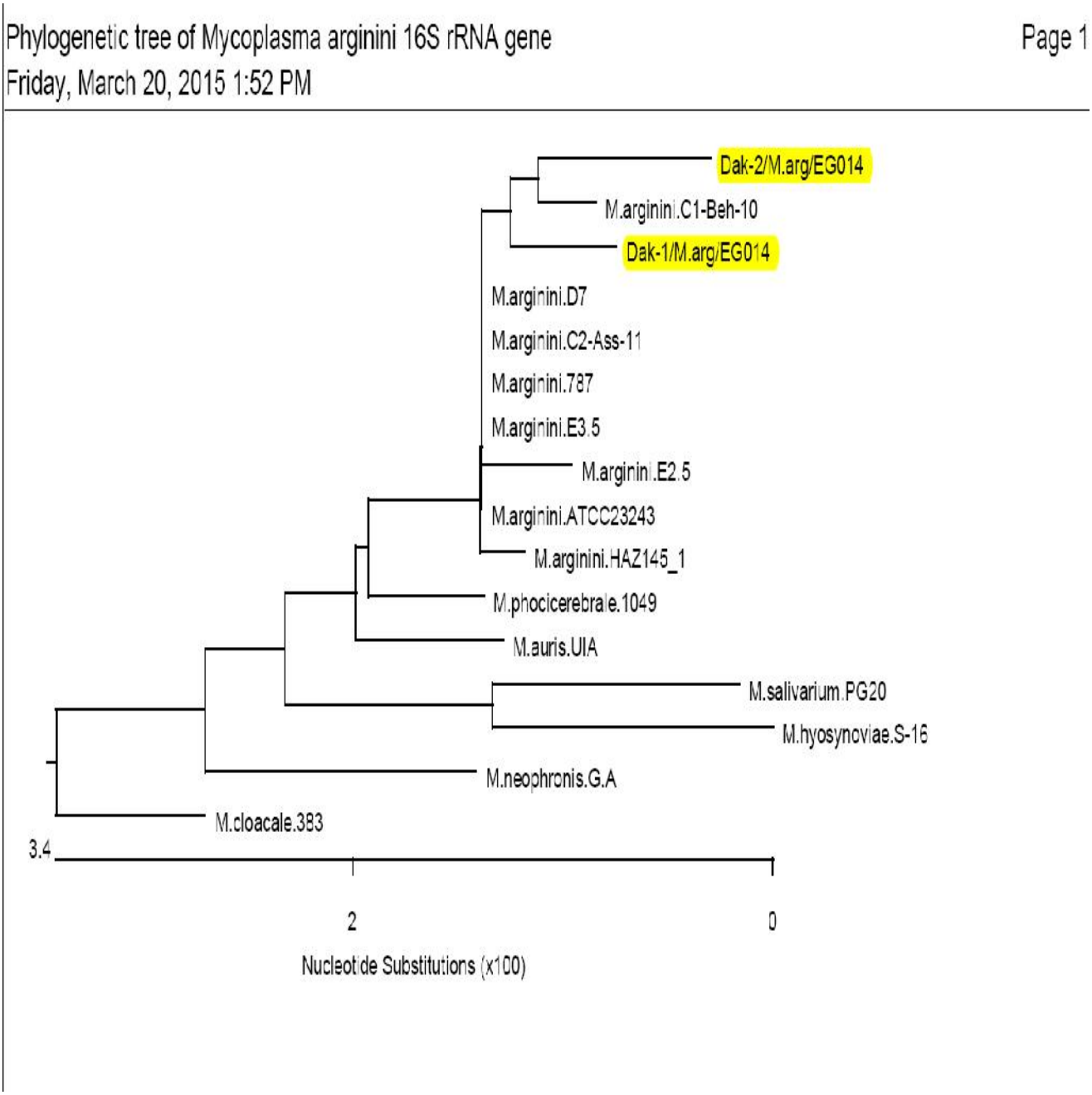

Fig (3). Phylogenetic tree of *Mycoplasma arginini* 16s rRNA gene from sheep and goat with other mycoplasma that were taken from the Gene Bank database 16s rRNA gene.

Nucletide sequence of mycoplasma

Discussion

Sheep and goats share their *Mycoplasma* flora with few exceptions, which can undoubtedly be explained by the close phylogenetic relationship of these two animal species. Mycoplasma infection in small ruminants is frequently observed, but more commonly in goats. Some of these mycoplasmas can cause severe and contagious diseases with serious economic impact. The most important mycoplasmas of ruminants are the pathogens of the *Mycoplasma mycoides cluster* **cottew *et al.,* (1987).**

In sheep, mycoplasmal pneumonia has been associated with *M. ovipneumoniae, M. capricolum* subsp. *capricolum, M. mycoides* subsp. *mcoides* LC and *M.arginini* **(Chaturvedi *et al.,* 1992) and (Ikhelo *et al.,* 2004).** *Mycoplasma arginini* appears to be the second most common species isolated and it is often present in mixed culture with *M. ovipneumoniae. Mycoplasma arginini* is less fastidious than *M. ovipneumoniae* and has the typical "fried-egg like" colony morphology which *M. ovipneumoniae* lacks **Goltz *et al.,* (1986)**. In our study we found that *M.arginini is* closely associated with sheep and goat pneumonia with percentage of isolation reached to 25% in all ages. The isolation of mycoplasmas is considered to be one of the most difficult tasks for diagnostic laboratories due to their inability to grow easily in laboratory media in spite of the great improvement in medium formulation **Waleed *et al.,* (2006)**, Although isolation and identification of *Mycoplasma* still an efficient Toole in the diagnosis of mycoplasma diseases. Multiple mycoplasmas may well co-exist in goat lungs and, in that case, only the strains which grow well will be recovered. Sheep are less susceptible to pulmonary mycoplasmoses than goats **Thiaucourt and Bolske (1996),** which is different with our results as the percentage of mycoplasma isolation was nearly equal in sheep and goat but none of the isolates could be identified as one of the mycoplasma mycoides cluster using PCR isolates were positive using 16S rRNA gene primer used for ruminant *Mycoplasma species* detection giving a clear band at 1000bp .

In spite of *M. arginini* is of less importance in pneumonia of sheep and goats; it is frequently isolated from their respiratory tract. This result is agreed with that mentioned by **Zaitoun (2001)** who was able to isolate *M. arginini* and *M. agalactiae* from cases of bronchopneumonia in sheep due to mycoplasma alone or coupled with bacterial agents. In this study a total of 160 Nasal swab were collected from sheep and goat suffer from respiratory manifestation for trails of mycoplasma isolation and identification, our result revealed that in Table (3) & Fig (1) Mycoplasma spp. Was isolated in 40 (25%) nasal swab samples out of 160 nasal swabs obtained from sheep and goat has respiratory manifestation., in sheep the percent of isolation were (25.32%) 20 isolate out of 79 nasal swab while the percent of isolation in goat were (24.69%) 20 isolate out of 81 nasal swab examined.

According to age the higher percent of isolation were found in age group (1- 2) year old sheep (50%) as 7 isolates out of 14 nasal swab examined, while in goat the higher percent of isolation were in age group (6-12 month old) by 47.83% (11 isolates out of 23 nasal swab examined. The lower isolation rates were found in adult sheep and goats (> 2 year old) by 15.38% in sheep and 24.69% in goat. **Ammar *et al* (2008)** isolated *Mycoplasma arginini* from sheep and goats in Cairo and Giza as 21.11 % and 35.29 % respectively. In this investigation, one mycoplasma species, *M. arginini*, were identified from the pneumonic lung tissues of sheep and goat rising in some farms at Dakkahlia governorate Egypt, by PCR technique and nucleotide sequences of the *Mycoplasma arginini* 16s rRNA. Polymerase chain reaction has been shown by others as an extremely sensitive and specific technique that allows direct detection of antigen and overcomes to cross-reactions of conventional tests. A comparison of the PCR technique with the microbiological culture, DNA fluorochrome staining, and hybridization techniques indi-

cated that the PCR is a rapid, sensitive, and efficient method **(Ball and Finlay, 1998, Van Kuppeveld *et al.*, 1994 and Gupta *et al.*, 2014).**

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العزل والتوصيف الجزيئي للميكوبلازما أرجينيني المعزولة من الأغنام والماعز المصابة بأعراض تنفسية في محافظة الدقهلية

د.دينا الشافعي *، د.نرمين عوض ، أ.د. ياسر النقر *** ، أ.د. عماد يونس ******

* أم. قسم الميكوبلازما – معهد بحوث صحة الحيوان
** زميل – كلية الطب البيطري جامعة المنصورة
***أستاذ أمراض الحيوان – كلية الطب البيطري – جامعة جنوب الوادي
****أستاذ الأمراض المعدية – كلية الطب البيطري - جامعة المنصورة

الملخص العربي

في هذه الدراسة تم جمع عدد ١٦٠ مسحات أنفية من الأغنام والماعز اللآتي تعاني من أعراض تنفسية. تم عزل الميكوبلازما أرجينيني لعدد ٤٠ (٢٥ ٪) من الأغنام والماعز. في الأغنام كانت نسبة العزل (٢٥.٣٢٪) ٢٠ من ٧٩ المسحات الأنفية ، بينما كانت النسبة في الماعز (٢٤.٦٩٪) ٢٠ من ٨١ مسحات الأنف التي تم فحصها. وفقا للسن وجدت نسبة العزل أعلى في الأغنام ذات الفئة العمرية (١- ٢) لسنة (٥٠٪) كما تم عزل ٧ من أصل ١٤ مسحة أنفية ، في حين الماعز كانت النسبة أعلى ة في الفئة العمرية (٦- ١٢ شهرا) ٤٧.٨٣٪ (١١ معزولة من ٢٣ مسحة أنفية تم فحصها. وكانت النسبة منخفضة في الأغنام الكبار والماعز (> ٢ العمرسنة) ١٥.٣٨٪ في الأغنام و٢٤،٦٩٪ في الماعز. تم تصنيف الميكوبلازما كما تم تسجيلها في بنك الجينات بإختبار أنزيم البلمرة المتسلسل وعمل التسلسل الجيني للحمض النووي.