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MYCOPLASMA OVIPNEUMONIA: ISOLATION AND MOLECULAR IDENTIFICATION IN DISEASED SHEEP FLOCK IN DELTA REGION, EGYPT

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ABSTRACT

Mycoplasma ovipneumoniae (M. ovipneumoniae) was isolated from nasal swabs obtained from sheep with respiratory manifestations in delta region, Egypt; wherein 31 sheep with different ages were suffer from nasal discharge, cough, pneumonia, keratoconjunctivitis in a flock of sheep containing 134 sheep. By mycoplasma culture, nine samples were positive from 31examined samples (29%). *M.ovipneumoniae* is detected in 33.33% (3 out of 9 isolates of mycoplasma) using PCR with specific primer. The high percentage of isolation found in more than one-year age group, strains were isolated from the nasal swabs and no detection from ocular swabs. Isolated *M. ovipneumoni* strain subjected to sequencing and was designated as NMD-EG016, which showed a 94.6% 16S-23S RNA intergenic spacer sequence identity with three USA strains (*M. ovipneumoniae*-2014-2278-10, *Mycoplasma.sp.* clone-10OR05 and *M.ovipneumoniae*-1992-6751-17) and >94.4with standard strain ATCC 29419.

Key words: *Mycoplasma ovipneumoni, Sheep, PCR.*

INTRODUCTION

Mycoplasma species are recognized for many diseases as arthritis, respiratory, eye lesions, genital disease and mastitis (Sharif and Muhammad 2009), Mycoplasma Diseases leads to major economic losses in small ruminant (Nicholas, 2002). Additionally, Mycoplasma ovipneumoniae *(M. ovipneumoniae), M. arginini and M. agalactiae* are the most important causes of sheep respiratory diseases (Lin *et al.,* 2008).

M. ovipneumoniae causes lethal pneumonia in sheep and goats as it is the infectious agent in ovine pleuropneumonia (Lin *et al.,* 2008; Dassanayake *et al.,* 2010). This organism is prevalent and highly contagious in almost every flock, causing major economic losses in the ovine industry worldwide. Compared to other pathogenic mycoplasmas, studies on *M. ovipneumoniae* are restricted by many aspects including the lack of the entire genomic sequence. This markedly hinders the understanding of the pathogenic mechanisms and the molecular basis of *M. ovipneumoniae* infection. (Minion *et al.,* 2004).

Using high quality samples in sensitive molecular diagnostic techniques led to identification of *M. ovipneumoniae* which previously ignored bacterium as a primary causative agent of pneumonia in bighorn sheep (Besser *et al.,* 2008, 2012a, b) more over When introduced into native bighorn sheep populations, outbreaks of polymicrobial pneumonia arise, sometimes resulting in high mortality in all age classes (Besser *et al.,* 2014). *M. ovipneumoniae* is also associated with mild and transient respiratory disease, usually in juveniles, in its normal domestic sheep and goat hosts (DaMassa *et al.,* 1992, Martin and Aitken 2000). However, several investigators have reported that *M. ovipneumoniae* infections in domestic sheep and goats can cause severe pneumonia, particularly when multiple strains are present (Parham *et al.,* 2006, Rifatbegovi, *et al.,* 2011).

Mycoplasma naturally need weeks to culture and also many serological tests are non-specific and insensitive as they are highly fastidious. Recently,

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many mycoplasma species have been detected by using PCR. Laura McAuliffe *et al.* (2005). Although *M. ovipneumoniae* is very important in sheep and goat industry, little genomic information is available. Therefore, this study was aimed to detect and sequencing the genome of *M. ovipneumoniae* to help further studies in the future.

MATERIALS AND METHOD

Study area and animals

This study carried out on a sheep flock containing 134 sheep in a private farm located at Dakahalia governorate in the north of delta region. Sheep were suffering from respiratory signs associated with ocular, nasal discharge, eye cloudiness, coughing and rise of body temperature during January 2016. The flock was grazing outdoors all day and at night was kept indoors. Sheep were allocated according to their age into, three groups 40 sheep (more than 1 year), 35 lambs (1-6) months old and 59 lambs (6 months – 1 year old), history of vaccination and epidemiological data of the flock were recorded.

1. Clinical examination

Clinical examination of sheep flock was done according to Kelly (1990).

Samples

Nasal and ocular Nasal swabs:

62 swabs (31 Nasal and 31 ocular swabs) were collected from the diseased sheep, classified as 10 nasal and 10 ocular swabs from lambs (1-6) months of age, 9 ocular and 9 nasal swabs from lambs of (7 months – 1 year), and 12 ocular and 12 nasal swabs from sheep more than 1 year old. The swabs were collected on PPLO broth and then transmitted on icebox as early as possible to Mycoplasma Department, Animal Health Research Institute, Dokki, Egypt for Mycoplasma isolation and identification.

2. Isolation of Mycoplasma:

Media used for cultivation and isolation of *Mycoplasma*:

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

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

c. 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).

3. Polymerase chain reaction (PCR):

- **Preparation of samples for DNA extraction (Yleana *et al.*, 1995),** One ml of outgrown suspension cultures was centrifuged for 10 min at 8 000rpm. The pellet was washed twice in 200 µl of phosphate buffered saline (PBS). The pellet was then suspended in 25 ml of H20, heated in a boiling water bath for10 min to break the cell membranes, rapidly chilled on ice then Centrifuged for taking supernatant.

- **Primer Selection.**

Table 1: Primers of Mycoplasma.

Target agent	Target gene	Primers sequences	Amplified segment (bp)	Primary den.	Amplification (35 cycles)				Reference
					Sec. den.	Ann.	Ext.	Final ext.	
M. ovipneumoniae	16S–23S intergenic spacer	GGAACACCTC CTTTCTACGG CCAAGGCATC CACCAAATAC	**402**	94˚C 5 min.	94˚C 30 sec.	58˚C 45 sec.	72˚C 45 sec.	72˚C 10 min.	Besser *et al.*, 2012b

Den.=denaturation, sec=secondary, Ann=annealing, Ext=extension

- **PCR amplification**

PCR amplification for Mycoplasma was performed in 50 µl reaction mixture consisting of 12 µl of 50 ng *Mycoplasma genomic* DNA, 25 µl of 2 x Master mix (Multiplex gen) VIVANTIS ,2 µl of 50 pmol of each primer, and 9 µl 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 electrophoresed 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.

4. Selected published sequences of 16S-23S intergenic spacer rRNA genes which were used in sequence analysis and phylogeny:

< *M.ovipneumoniae*-ATCC 29419 gbAY753216.1
< *M.ovipneumoniae*-2014-2278-10 gbKU986496.1washintagon USA
< *Mycoplasma.sp.clone*-10OR05 gbJN857916.1 USA
< *M.ovipneumoniae*-1992-6751-17 gbKU986493.1USA
< *M.ovipneumoniae*-2006-7402a gbKU986494.1
< *M.ovipneumoniae*-1987-3722-1 gbKU986492.1
<*M.ovipneumoniae*-2009-11512-3 gbKJ551511.1
< *M.ovipneumoniae*-10-698-1 gbHQ615162.1
<*M.ovipneumoniae*-2014-7753-3 gbKU986495.1

5. Sequence and phylogenetic analysis

DNA Sequencing of ISR gene was conducted in both directions and a consensus sequence of 422 (gbpaper450) bp was used for nucleotide analysis. The original sequence was trimmed to remove vague nt. sequences usually exist in the beginning of the sequencing reaction. Partial DNA sequences was submitted to GeneBank database and obtained accession number; KY562849. Identification of homologies between nucleotide sequence of the studied *Mycoplasma ovipneumoniae* and others published in GenBank was done using BLAST 2.2 search program (National Center for Biotechnology Information "NCBI" http://www.ncbi.nlm.nih.gov/). Comparisons of the obtained nucleotide sequence with other Mycoplasma sequences that published in GenBank 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 (Thompson *et al.*, 1994). MegAlign calculated sequence divergence and identity percentages.

RESULTS

I. Clinical examination

Examined sheep showed, nasal and ocular discharge, ocular cloudiness and keratoconjunctivitis in addition to coughing and rise of body temperature, these signs were obvious in the age group less than 1 year old (1-6 months of age) Table (2). Fig (1).

Fig (1): Photo-showing sheep suffer from mucopurullent nasal discharge

Table 2: Clinical examination of sheep flock.

Age group	Clinical examination			Main clinical signs
	Total	Diseased	%	
1-6 months	**35**	**10**	**28.57**	Ocular discharge, nasal discharge, coughing and rise of body temperature
7 - 12 months	**59**	**9**	**15.25**	Ocular discharge, eye cloudiness, nasal discharge, coughing and rise of body temperature
More than 1 year months	**40**	**12**	**30**	Ocular discharge, eye cloudiness, nasal discharge, coughing
Total	**134**	**31**	**23.13**	

II. Isolation of *Mycoplasma spp*. from nasal and ocular nasal discharge.

Mycoplasma spp. was isolated from 9 out of 31 examined nasal swabs by (29%) while all examined ocular swabs were negative for isolation table (3)**.** The highest isolation rate were recorded in more than one-year age group sheep by 41.67%

Table 3: Isolation rate of *Mycoplasma spp* from nasal and ocular swabs in relation to age.

Age group	Nasal swabs			Ocular swabs		
	Samples (No.)	Positive	%	Samples (No.)	Positive	%
1-6 months	10	2	20	10	0	0
7 months-1year	9	2	22.22	9	0	0
More than 1 year	12	5	41.67	12	0	0
Total	**31**	**9**	**29**	**31**	**0**	**0**

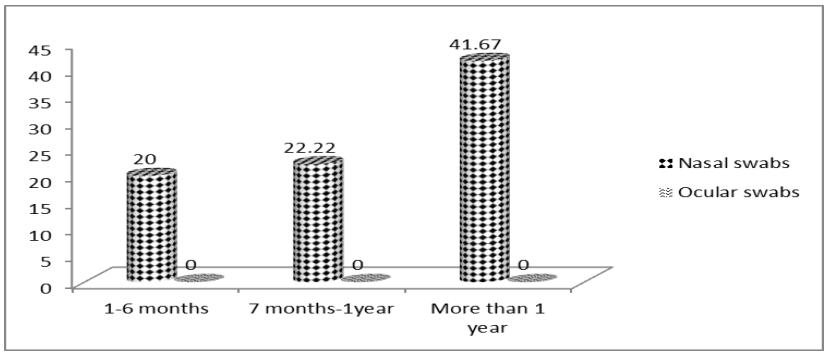

Fig (2). Percent of *Mycoplasama ovipneumoni* isolation from nasal, ocular swabs of sheep in relation to age.

P.C. R Detection of *M. ovipneumonea*

Of the 9 cases found positive Mycoplasma spp, 3 isolates were positive with specific species primers for *M. Ovipneumonea*. The bands obtained were 402 bp. Fig (2). The three positive isolates of M. ovipneumoniae were recognized in 1 - 6-month age group, 2 isolates and one isolates in 7 – 12-month age group and not detected in age group over than 12 months of age.

Table 4: Identification of isolated *Mycoplasma* spp. using PCR.

Total isolates	*Mycoplasma ovipneumoni*	%	Another Mycoplasma	%
9	**3**	**33.33**	**6**	**66.66**

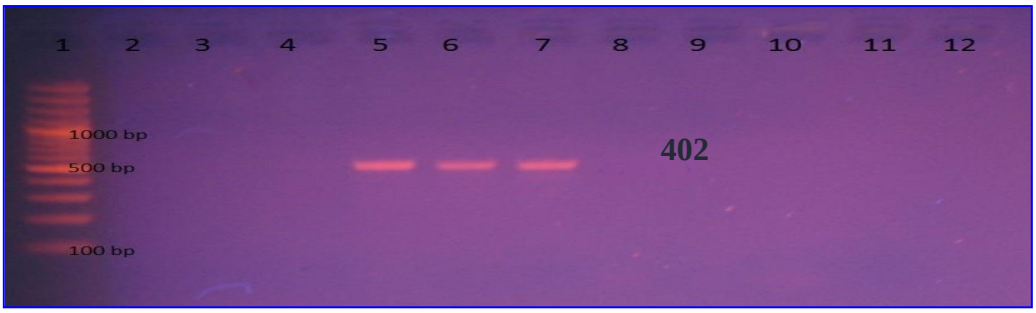

Fig (3): Gel electrophoresis of PCR products of *M. ovipnumoniea*16S-23S intergenic Spacer. 1: VC100BP Puls DNA Ladder, Lane 5, 6, 7 The amplified products prepared from positive Nasal Swabs of diseased sheep, Lane 2: negative control, Lanes 3, 4 &8-11 negative results.

As the presence of *M.ovipneumoniae* was confermed, the purified PCR product for one isolate exposed to sequencing and analysis. The sequence was submitted to NCBI GenBank (Accession number KY562849.). Phylogenetic tree was built based on the ISR gene sequence of one *M. ovipneumoniae* isolate with standard strain and others *M. ovipneumoniae* published in GenBank (Fig.4). The isolate showed 94.6% sequence identity with *M. ovipneumoniae*-2014-2278-10, *Mycoplasma spp* - clone-10OR05 and M. ovipneumoniae1992-6751-17 (from USA). Moreover, sequences of the isolate showed >94.4% identity with that of the standard strain (*M. ovipneumoniae*-ATCC).

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11		
1		92.0	91.7	90.8	92.6	94.4	94.6	94.6	94.6	92.9	94.4	1	NMD-EG016
2	8.5		99.7	99.7	94.1	96.3	96.5	96.5	96.5	95.8	96.5	2	M.sp-clone186759
3	8.8	0.3		100.0	94.3	96.5	96.8	96.8	96.8	96.0	96.8	3	M.ovipneumoniae-10-698-1
4	9.8	0.3	0.0		93.7	95.1	95.3	95.3	95.3	94.9	95.3	4	M.ovipneumoniae-2009-11512-3
5	7.8	6.2	5.9	6.6		95.5	95.8	95.8	96.0	96.3	95.5	5	M.ovipneumoniae-2014-7753-3
6	5.8	3.8	3.5	5.1	4.6		99.5	99.5	99.5	97.5	99.5	6	M.ovipneumoniae-ATCC
7	5.6	3.6	3.3	4.9	4.4	0.5		100.0	99.5	97.5	99.5	7	M.ovipneumoniae-2014-2278-10
8	5.6	3.6	3.3	4.9	4.4	0.5	0.0		99.5	97.5	99.5	8	Mycoplasma.sp.clone-10OR05
9	5.6	3.6	3.3	4.9	4.1	0.5	0.5	0.5		97.4	99.5	9	M.ovipneumoniae-1992-6751-17
10	7.5	4.4	4.1	5.3	3.8	2.6	2.6	2.6	2.6		97.5	10	M.ovipneumoniae-2006-7402a
11	5.8	3.5	3.3	4.9	4.6	0.5	0.5	0.5	0.5	2.6		11	M.ovipneumoniae-1987-3722-1
	1	2	3	4	5	6	7	8	9	10	11		

Divergence

Figure (4): Sequence identity and divergence between various isolates of *M.ovipneumonaiea*

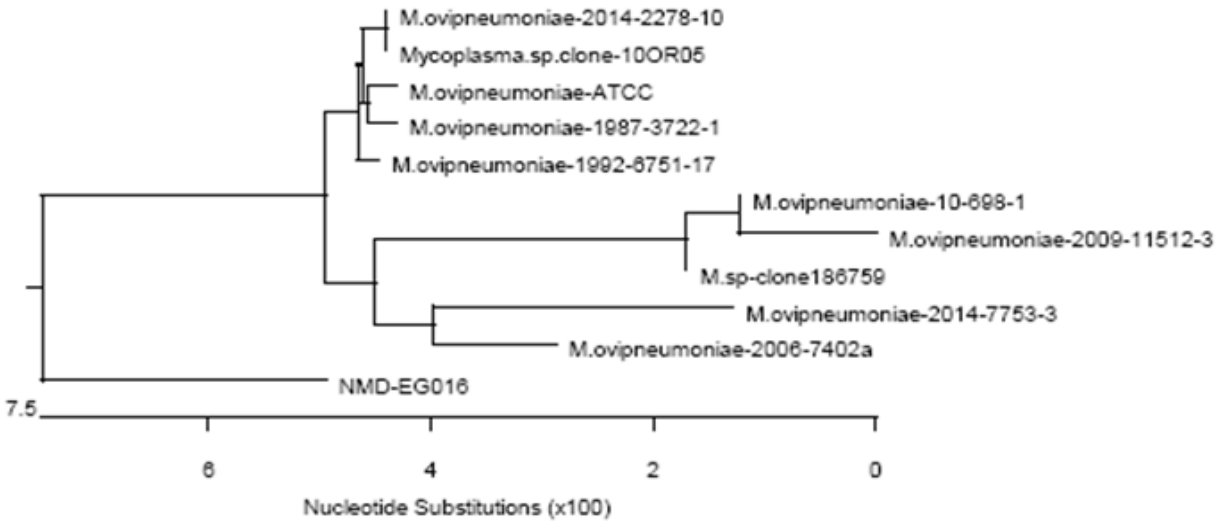

Figure (5): Phylogenic analysis of *M.ovipneumonaiae* isolates based on ISR sequence.

DISCUSSION

Mycoplasma with their extreme antigenic variation is concerned؛ mycoplasmas have complex mechanisms enabling them to evade the immune system causing several clinical symptoms, leading to significant economic effect on production of small ruminants Kumar *et al.* (2011).

M. ovipneumoniae considered the greatest mycoplasmas involved in sheep respiratory diseases. Primary infection with *M. ovipneumoniae* may predispose sheep to the lower respiratory tract infection by another organisms, Nicholas *et al.* (2008). This agent is frequently isolated from pneumonic sheep.

In this study, clinical examination of sheep flock containing 134 sheep revealed that, 31 sheep suffered from nasal and ocular discharge, ocular cloudiness and keratoconjunctivitis in addition to coughing and rise of body temperature, the reported clinical signs agree with Kumar *et al.* (2012), who mentioned that pneumonia accompanied by keratoconjunctivitis, mastitis, abortions، and arthritis is commonly observed in mycoplasma syndrome.

These signs were obvious in the age groups less than one year old. Table (2). Fig (1), the age group more than one year, has the high rate of respiratory Manifestations, Suzanna Bell (2008) reported that, acute form of mycoplasma appear in young lambs while a chronic infection is often found in older lambs and adults also, Elnaker *et al.* (2017) found that high mortality rate in sheep less than one year related to *M.ovipneumonaie* which likewise with Beseer *et al.* (2008).

The result of mycoplasma isolation Table (3) Fig (3) revealed that 9 isolates of mycoplasma by 29% from nasal swabs while no isolation from ocular swabs which nearly similar with result of Chinedu A. Akwuobu *et al.* (2014) who study the prevalence of Mycoplasma species in small ruminants in Nigeria and found that 25.8% of 508 examined small ruminant nasal swab were identified by PCR/DGGE

as *Mycoplasma spp*, also Ayşe Kılıc *et al.* (2013) found that 37.03 % of the isolates were mycoplasma spp by using culturing, and in our study, the high rate of isolation in age group more than one year was 41.67%.

Of 9 cases found positive for *Mycoplasma spp*, 3 isolates existed positive with species-specific primers for *M. Ovipneumonea*, Fig (2). The three positive isolates of *M. ovipneumoniae* were recognized in 1 - 6 months age group, 2 isolates and one isolates in age 7 - 12 months and not detected in age over than 12 months, this approve the suggestion of the main role of *M. ovipneumonaie* in sever pneumonic cases which agree with Besser *et al.* (2008) who informed that *M. ovipneumoniae* was identified as a main member of the pneumonic lung flora in lambs with early lesions of bronchopneumonia also Chinedu A. Akwuobu *et al.* (2014) reported *M. ovipneumoniae*, and *M. mycoides subsp. capri* among the important pathogenic Mycoplasma species for small ruminant. Massimo Giangaspero *et al.* (2012) found 26% of the isolates were seropositive to *M. ovipneumoniae*, from the other hand slightly higher percent of detection was noticed by Ayşe Kılıc *et al.* (2013), 59.37 % of mycoplasma isolates were *M. ovipneumonaie* recognized by PCR, Kumar *et al.* (2001) noted that molecular detection of Mycoplasma species based on different set of primers was used to identify different species. Sequencing of one strain referred to presence of >94.6 identity to two USA strains and >94.4 to standard strain which give attention to further studies especially after detection of *M. ovipneumonaie* from deferent flocks in Egypt producing serious pneumonic form which leads to high mortality rate.

Our results underscore the need to Supplementary work is necessary, both to clarify the epidemiology of mycoplasma infection in sheep, and evaluation of vaccination against Mycoplasma of mycoplasma infections in sheep.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests in the publication of this paper.

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الميكوبلازما اوفينيموني :عزل وتصنيف جزيئي في قطيع من الأغنام المريضة في منطقة الدلتا ، مصر

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تم عزل الميكوبلازما اوفينيموني من مسحات الأنف التي تم الحصول عليها من اغنام ظهر عليها اعراض تنفسيه في منطقة الدلتا ، مصر ، حيث كان ٣١ من الاغنام من أعمار مختلفة يعانون من إفرازات أنفية. كحه .التهاب رئوي والتهاب في العين في قطيع يحتوي على ١٣٤راس من الأغنام. تم عزل الميكوبلازما بنسبه ٢٩% (٩من ٣١ عينه) باستخدام الطرق التقليديه لعزل الميكوبلازما. تم تحديد وجود٣٣,٣٣% من عزلات الميكوبلازما كميكوبلازما اوفينيموني (٣من اصل ٩) باستخدام اختبار سلسة البلمره التفاعليه.كانت اعلي نسبة عزل للميكوبلازما اوفينومني في سن اقل من ٦ شهور وكلها من المسحات الانفيه.تم عمل التسلسل الجيني لواحد من عزلات الميكوبلازما اوفينيموني وتم تعريفه باسم NMD-EG016 ورقم تسجيلي KY562849 والتي اوضحت نسبة تطابق تصل الي ٩٤,٦% مع ثلاث عزلات من الولايات المتحده الامريكيه (M.ovipneumoniae-2014-2278-10, Mycoplasma.sp.clone-10OR05 and M.ovipneumoniae-1992-6751-17) باستخدام نفس التسلسل الجيني ١٦ S-23S RNA intergenic spacer وكذلك حوالي ٩٤,٤% مع سلالة قياسية ATCC 29419 .