Studies on respiratory affections in calves due To Mycoplasma in Dakahlia and Sharkia Governorates.

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Abstract

This study was carried on calves suffering from respiratory signs, fever, sometimes anorexia with age ranged from 2 month to 2 years. Eighty one samples were collected at the time of initial treatment (69 nasopharyngeal swabs and 12 lung tissues) from two main Governorates Dakahlia and Sharkia. The incidence of Mycoplasma isolation was 16.7% from DaKahlia and 26.7% from Sharkia. Six isolates were identified as *Mycoplasma bovirhinis*,five isolates were *Mycoplasma arginini* and seven isolates were *Mycoplasma bovis* from two big farms in Sharkia Governorate. PCR test was used to confirm the isolation by using the common gene 16SrRNA for ruminant Mycoplasmas which gave characteristic band at 1000bp.M.bovirhinis was confirmed using 16SrRNA which gave specific fragment at 316bp, while *Mycoplasma bovis* was confirmed using VsPA gene which gave specific fragment ranged between 342-500 bp. depending on the locality of isolation. Sensitivity test for some Mycoplasma isolates using MIC method revealed that Draxxin (Tulathromycin) has the lowest MIC for both *Mycoplasma bovirhinis* and *Mycoplasma arginini* with range (0.0048-0.007), while Enrofloxacin has the lowest MIC against *Mycoplasma bovis* (0.014).

Key words: *Mycoplasma bovis, Mycoplasma bovirhinis, Mycoplasma arginini, Tulathromycin.*

Introduction

Bovine respiratory disease (BRD) complex is a major disease of calves (**Virtala *et al.,* 1996** and **Duchemin, 2004).** Clinical symptoms typically appear after a stressful event such as transportation and grouping. BRD is a multifactorial disease. The role of class mollicutes specially *Mycoplasma bovis* is an active agent in cases of bovine respiratory disease (BRD) is poorly defined. *Mycoplasma bovis* is the predominant pathogen isolated from the middle ear of calves suffering from otitis media (**Haines *et al.,* 2001** and **Sharter *et al.*, 2002)**. Multiple *mycoplasma* species as *Mycoplasma bovirhinis*, *Mycoplasma dispar*, *Ureaplasma diversum* and even *Mycoplasma canis* have been isolated from respiratory healthy calves, **(Virtala *et al*., 1996** and **Duchemin, 2004)**. *Mycoplasma bovihrinis* is a troublesome agent as a secondary invader in respiratory disease, because it is the most commonly isolated from the nasal cavity of cattle with respiratory disease, **(Gourlay and Howard, 1979)**. The role of these organisms as primary or secondary pathogen unclear, the economic impact of mycoplasmosis whoever has been estimated to be significant in the beef industry due to weight loss and health consequences **(Lamm *et al*., 2004)**.

Franco *et al.* (2004), recorded that bacteria from class mollicutes specifically *Mycoplasma bovis* has been associated with pneumonia in cattle**,** and these organisms are frequently identified from chronic respiratory disease cases**, (Thomas and Ball, 2002**, **Wiggins *et al.,* 2007)**. In Europe, it is responsible for at least a quarter to a third of all calf pneumonia, **(Lamm *et al.*, 2004)**. Pneumonia is a major cause of economic losses because of decreased production, high levels of mortality and morbidity and increased veterinary and labor costs**, (Ganaba *et al*., 1995)**. Moreover calves experienced pneumonia at early age might have severe depression in the production capabilities

in the future, **(Sayed and Zaitoun, 2009)**. Previous research documents that these organisms may be found in the nasal discharge from healthy and chronically ill animals **(Nicholas and Ayling, 2003)**.

So the purpose of this study was to study the role of mycoplasmosis as a cause of respiratory infection in calves by using culture procedures and polymerase chain reaction (PCR) to determine its prevalence in field cases with BRD at the time of initial treatment.

Material and Methods

Samples:

A total of 81 samples were collected from calves, **69** deep nasal swabs and **12** lung tissues, (**36** from Dakahlia Governorate and **45** from Sharkia Governorate) with age ranged from 2 months to 2 years, these animals suffering from respiratory signs as cough, dyspnea and nasal discharge, some times fever with different degrees of anorexia.

Culture procedures (Sabry and Ahmed, 1975):

The collected samples were transported to PPLO broth and incubated at 37^{o}C for 24 hours; the incubated broth samples were platted on PPLO agar plates under reduced oxygen tension in humidified candle jar and examined for mycoplasma colonies after 48 hours then daily up to 7-10 days for fried egg colonies.

Biochemical identification:

1-Digitonin test (Frundt *et al.*, 1973).

This test was made to differentiate between genus mycoplasma and genus acholeplasma depending on sterol requirement for growth, the cultivated plates were incubated in suitable condition and examined for inhibition zone, mycoplasmas are digitonin positive showing marked inhibition zone, while acholeplasma are digitonin negative.

2-Glucose fermentation test (Erno and Stipkovits, 1973).

The suspected cultures were inoculated into glucose medium with control and incubated at 37 ºC, if the color changed from red to yellow or orange is considered positive.

3-Arginine deamination test (Erno and Stipkovits, 1973).

The purified culture was examined to its ability to hydrolyze arginine, if the color changed from red to pink is considered positive.

4-Identification by PCR:

The biochemically characterized mycoplasma isolates were investigated by PCR test for identification.

DNA extraction:

1-Extraction of chromosomal DNA (Fan *et al.*, 1995):

Five ml quantity of overnight incubated culture from each Mycoplasma isolate was centrifuged in a micro-centrifuge at 13000 rpm.for 3 minutes. The cell pellets were washed twice in 100 µl of 150 mM phosphate buffered saline (PBS, pH 7.2) and suspended in 25 µl PBS. The cell suspension was heated directly at 100 ºC for 10 minutes in a heat block and then put on ice for 10 minutes. Finally, the cell suspension was centrifuged for 3 minutes, and chromosomal DNA was collected and stored at 4^{o}C.

Oligonucleotide primers encoding 16S common gene for ruminant Mycoplasma and VspA specific gene for *Mycoplasma bovis* **(Alberto *et al.*, 2006),** while *Mycoplasma bovirhinis* was 16SrRNA specific gene as described by **(Kobayashi *et al.*, 1998**).

Primer of 16SrRNA common gene.

16Smuniv F: 5'-AGA CTC CTA CGG GAG GCA GCA-3'.

16Smuniv R: 5'-ACT AGC GAT TCC GAC TTC ATG-3'.

Primer of 16SrRNA gene of *Mycoplasma bovirhinis*

Mbr F: 5'-GCT GAT AGA GAG GTC TAT CG-3'

Mbr R: 5'-ATT ACT CGG GCA GTC TCC-3'

Primer of Vsp A specific gene of *Mycoplasma bovis*

MYB F: 5'-CTT GGA TCA GTG GCT TCA TTA GC-3'.

MYB R: 5'-GTC ATC ATG CGG AAT TCT TGG GT-3

The sequence of 16SrRNA gene was amplified a product at 1000 base pair (bp) while a specific gene of *Mycoplasma bovis* (VspA) was amplified a product at 420 bp. Primers were pre-

pared by Sigma Company.

PCR procedures (Alberto *et al*., 2006):

The PCR reaction mixture (total volume 50µl.PCR was performed on a progene programmable thermal controller (UK).

2- Cycling procedures:

The amplification was performed by heating the sample for 9 minutes at 94 °C then 35 cycles of denaturation for 45 sec. at 94 °C, annealing for 1 minute at 60 °C, and extenuation step for 1.5 min. at 72 °C, a final extenuation step at 72°C held for 5 min. The analysis of PCR products was performed by using 10µl of amplified PCR product, mixed with 2µl loading buffer and electrophoresed through 1.5% agarose gel and DNA was visualized by UV fluorescence after ethidium bromide staining and then photographed.

3- Antimicrobials: The antimicrobials used and the form of which they are available and

Table (1). Antimicrobial drugs used for MIC determination.

Drug	Group	Form used	Source
Draxxin	Macrolide	25% Solution	Pfizer USA
Enrofloxacin	Quinolones	10% Solution	Alex.Vet.Egypt
Noflor	Florofenicol	10% Solution	Schering plough
Pan terramycin	Tetracycline	3% Solution	Pfizer Egypt
Tylocin 200	Macrolide	20% Solution	Avico Jordan

Sterile stock solution:

Sterile stock solutions containing 160µg/ml were prepared from each drug in distilled water and stored at 4°C or used freshly.

Titration of Mycoplasmas:

For each Mycoplasma isolate, the number of color changing units (ccu) was determined by the method described by **Tylor- Robinson (1983)**. A titer of 10^3-10^4 cfu/ 0.2ml was required for test proper **(Senterfit, 1983).**

Determination of minimal inhibitory concentration (MIC):

The test was performed in duplicate exactly as described by **Senterfit (1983).** The antimicrobials were tested in serial two fold dilutions at conc. ranging from 20 to 0.009 µg/ml.

End point reading:

The MIC is the lowest concentration of the antimicrobial that completely prevent a color change. This typically occurred after days. For comparison a final reading taken after 14 days incubation **(Whither *et al.,* 1983).** Results were expressed in µg/ml of the active material.

Results

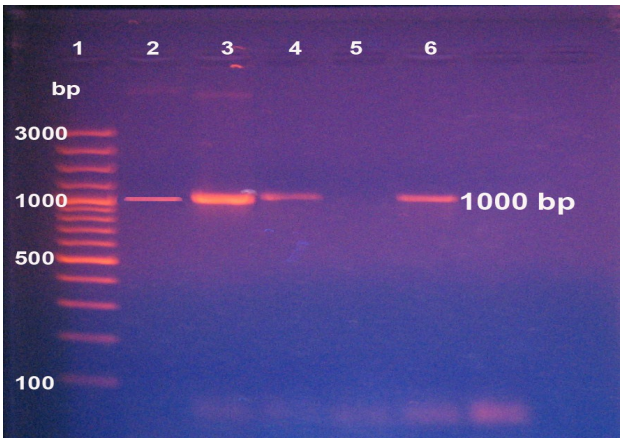
Eighteen mycoplasma isolates and 3 aholeplasama species were isolated and identified from calves suffering from respiratory signs in Dakahlia and Sharkia governorates as in Table (2). Six isolates from Dakahlia (16.7%) (4 *Mycoplasma bovirhinus* and 2 *Mycoplasma arginine*) and twelve isolates (26.7%) from Sharkia (2 *Mycoplasma bovirhinus*, 3 *Mycoplasma arginine* and 7 *Mycoplasma bovis*).

Table (2). Primary isolation, biochemical identification and PCR results of mycoplasma isolated from calves.

Governorate	samples Type	No.of Tested sample	Digitonin		Isolation %	Biochemical characterization						Identification		
						glucose		Arginin		Film&spot				
			+ve	-ve		+ve	-ve	+ve	-ve	+ve	-ve	*M.-bovirhinis	**M.Argi-nini	*** M.bovis
El Dakahlia	Nasal swab	36	6	2	16.7%	4	-	2		0		4	2	0
El Sharkia	Nasal swab	33	10	1	30.3	2	-	1		7		2	1	7
	Lung tissue	12	2	-	16.7	0	-	2		0		0	2	0
Total		81	18	3	22.2	6		5		7		6	5	7

* *Mycoplasma bovirhinis*
** *Mycoplasma arginini*
* *** *Mycoplasma bovis*

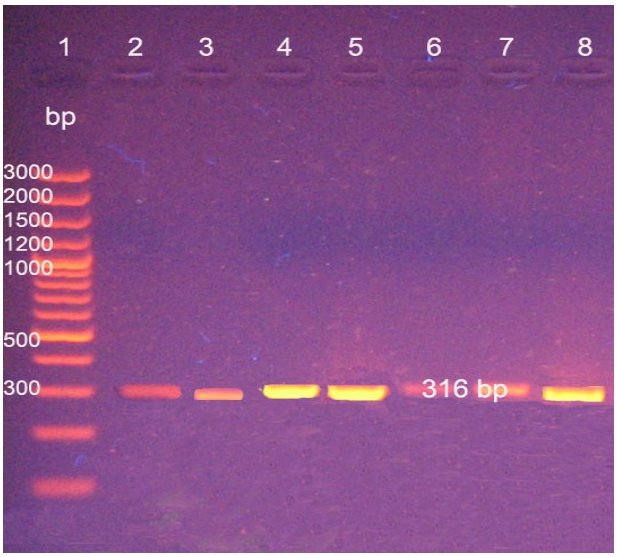
Photo (1). Shows the electrophoretic pattern of common primer of Mycoplasma (16SrRNA) isolated from calves.



Lane: (1).100bp DNA ladder
Lane: (2). control positive
Lane: (5). control negative
Lane: (3, 4, and 6). *Mycoplasma species* isolated from calves.

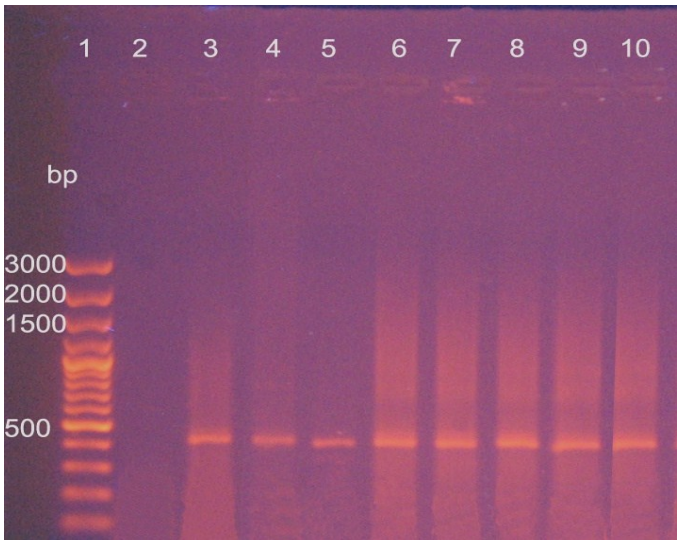
This photo shows, the characteristic fragment of mycoplasma at 1000bp where four isolates characterized from diseased calves were subjected to examination by the common primer of ruminant (16SrRNA).

Photo (2). Shows the electrophoretic pattern of *Mycoplasma bovirhinis* by 16SrRNA specific gene.



Lane (1). 100bp DNA ladder
Lane (2). positive control
Lane (3-8). Field isolates from calves
This photo shows, the characteristic fragment of *Mycoplasma bovirhinis* at 316 bp where six isolates characterized from diseased calves were subjected to examination by the 16SrRNA specific primer.

Photo (3). Shows the electrophoretic pattern of *Mycoplasma bovis* by VspA gene.



Lane (1). 100bp DNA ladder
Lane (2). Negative control
Lane (3). positive control for *Mycoplasma bovis*
Lane (4-10). Field isolates from calves
This photo shows, the characteristic fragment of *Mycoplasma bovis* at 420 bp where seven isolates characterized from diseased calves were subjected to examination by the VspA gene**.**

Table (3). In vitro activities of five antimicrobials against Mycoplasma isolated from calves.

Mycoplasma isolates	Minimal inhibitory concentration				
	Draxxin	Enrofloxacin	Florofenicol	Oxytetracyclin	Tylosin
M.bovirhinis (2 isolate)	0.0048	0.048	0.048	0.058	0.039
M.arginini (3 isolate)	0.007	0.05	0.05	0.05	0.039
M.bovis (7 isolate)	0.078	0.014	0.156	0.078	0.039
C.Max µg (mcg)/mL	6.9	1.88	2	54.58	4.2

C-max: Maximum observed plasma concentration

Application of different antimicrobials as shown in table (3) and comparison of MIC to the C -max revealed that most of antibiotics used gave MIC less than C-max of the same antibiotics and we divided the activity of the antibiotics in relation to C-max. Draxxin has the lowest MIC for both Mycoplasma bovirhinis and mycoplasma arginine (0.0048-0.007), while, enrofloxacin has the lowest MIC for mycoplasma bovis (**0.014 µg**/ml) than other antimicrobials used in this study.

Discussion

Several species of mycoplasma may be isolated from calves with pneumonia but only few of these species are considered pathogenic .Respiratory pathogenic mycoplasma spp. includes *M.dispar, M. bovis, M.bovirhinis, M. bovigenitalium, Ureaplasma diversum*, **(Nicholas *et al.*, 2000**). **Gourlay and Howard (1979**), reported isolation of Mycoplasma from lungs of 30% of calves that died with sever pneumonia and 60% of calves with subclinical pneumonia, these results agree with our results where we were able to isolate mycoplasma from pneumonic lungs by 16.6% and from nasopharyngeal swabs by 16.7% in Dakahlia and 30.3% in Sharkia Governorates.

In this study, *M. bovirhinis* were identified in 7.4% of the samples examined and *M. arginine* by 6.17% while *M. bovis* could be isolated and identified in 8.64 %.

Prevalence of *M.bovis* is often underestimated in both beef and dairy herds because of the difficulties in diagnosis and the variety of clinical signs associated with this infection and disease caused by this pathogen**, Nicholas and Ayling (2003).** Bacterial isolation which was considered the gold standard test for mycoplasma infections, is difficult and time consuming because of the fastidious nature of mycoplasmas, which require 1) specialized media and incubation conditions; 2) extended culture time as a result of the slow growth of the organism (up to 14 days for completion); and 3) additional testing for speciation. **Khodakaram and Lopez (2004)**. Other respiratory pathogens are often isolated more easily and quickly, and attempts at detection and identification of *M. bovis* may not be pursued**, (Haines *et al.*, 2001** and **Freundt, *et al*., 1973).**

Transmission of *M.bovis* in respiratory secretions is considered important in the epidemiology of infection *M.bovis* may be transmitted in respiratory secretions via aerosols, nose to nose contact, or indirectly via feed, water, housing, or fomites. The importance of aerosols in calf to calf transmission of *M.bovis* is unknown, but *M.bovis* has been isolated from air in barns containing diseased calves (**Jasper *et al.*, 1974**), and calves can be experimentally infected by inhalation of *M.bovis*, (**Nicholas *et al*., 2002).** This can explain the results of isolation of *M. bovis* from two farms in Sharkia Governorate.

A test based on PCR targeting 16SrRNA gene with mycoplasma specific primers and separation of the product according to the primer sequence using denaturing gradient gel electrophoresis (DGGE) has recently been applied for the detection of mycoplasma in samples (**Laura *et al.*, 2003** and **Blackburn *et al*.,**

2010).

In our study we used three PCR assays, a common 16SrRNA gene can detect ruminant mycoplasma from calves as in photo 1, while,16SrRNA specific gene of *M.bovirhinis* (**Kobayashi *et al*., 1998**) gave specific band at 316 bp, this agree with our results as in photo 2. Using VspA gene for *M. bovis* we could identify seven strains as in photo 3, this agree with (**Alberto *et al*., 2006),** who could isolate and identify ***M.bovis*** from cattle suffering from kerato-conjunctivitis.

Mycoplasma is very difficult to treat because many commonly used antibiotics do not work well. Penicillin kills bacteria by destroying the cell wall. Since *Mycoplasma* does not have a normal cell wall, these antibiotics are ineffective in treating it. Oxytetracycline has produced mixed results in treating *Mycoplasma*, in one study 50 percent of *M. bovis* isolates were resistant to oxytetracycline. Tulathromycin is the only drug approved for Mycoplasma, and in one study, was the drug most likely to be effective **(Godinho *et al*., 2005).** In our study Tulathromycin was very effective in vitro on *M.bovirhinis* and *M.arginine* while oxytetracycline has the highest MIC on these strains. Enrofloxacin has the lowest MIC for *M. bovis* (**0.014 µg**/ml) than other antimicrobials used in this study, this agree with the results of **Hannan *et al*. (1997**) who found that enrofloxacin has the lowest MIC on *M.bovis* isolates.

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دراسات عن الاصابات التنفسية بالميكوبلازما فى العجول فى محافظتى الدقهلية و الشرقية.

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الملخص العربى

تم عمل هذه الدراسة على العجول التى تعانى من أعراض تنفسية و ارتفاع فى درجة الحرارة وفقدان فى الشهية وكانت الاعمار تتراوح بين شهرين الى سنتين ذلك قبل بدأ اعطاء العلاج حيث تم تجميع عدد 81 عينة (69 مسحة من الانف و البلعوم و 12 عينة من أنسجة من الرئتين) من محافظات الدقهلية و الشرقية-مصر و تم عزل الميكوبلازما من محافظة الدقلهلية بنسبة 16.7% و من محافظة الشرقية بنسبة 26.7% حيث تم تصنيف 6 معزولات من الميكوبلازما بوفيرينس,5 معزولات من الميكوبلازما أرجينينى و 7 معزولات من الميكوبلازما بوفيس و كانت معزولات الميكوبلازما بوفيس من مزرعتين فقط من محافظة الشرقية. تم تصنيف المعزولات بالاختبارات البيوكيميائية و اختبار أنزيم البلمرة المتسلسل (PCR) باستخدام جين ال 16SrRNA و من خلاله تم تاكيد العزل والذى يعطى حزمة مميزة عند 1000bp و تم تاكيد تصنيف معزولات الميكوبلازما بوفيس باستخد م جين VspA و الذى يعطى حزمة مميزة عند 400bp . باجراء اختبار الحساسية لبعض المعزولات الممثلة بطريقة أقل تركيز مثبط MIC و مقارنته باعلى تركيز للمضاد الحيوى فى جسم الحيوان C-MAX وجد أن عقار الدراكسين(التولاثرومايسين) أكثر تاثيرا على ميكوبلازما بوفيرينس و ميكوبلازما أرجيننى باقل تركيز مثبط ≤ **0.0048** و أقل من C-MAX لنفس المضاد , بينما كانت معزولات الميكوبلازما بوفيس أكثر تاثرا بعقارالانروفلوكساسين باقل تركيز مثبط ≤ **0.014**و أقل من C-MAX لنفس المضاد.