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Molecular investigation of respiratory infections syndrome caused by *Mycoplasma bovis* and *Pasteurella multocida* in cow calves

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Abstract

Bovine respiratory disease (BRD) caused by various mycoplasmal, bacterial, viral and mycotic agents is a major health problem of cattle worldwide.

Mycoplasmas are generally host-specific, and most diseases associated with animal mycoplasmosis are chronic with high morbidity and low mortality. *Mycoplasma bovis* is traditionally known as a major contributing etiological agent in enzootic pneumonia of calves, mastitis of cows and arthritis of young and adult bovine animals. Also Pasteurella multocida is considered a leader of organisms causing respiratory distress in calves.

Bacteriological examination of 240 samples (nasal swabs, lungs, and tracheas) was performed to determine the prevalence of *Mycoplasma bovis* and *Pasteurella multocida* in relation to clinical signs of respiratory disease in cow calves and apparently healthy ones. *P. multocida* showed higher incidence in both apparently healthy and diseased animals (6.6%, 16.6% respectively) than *M. bovis* (5%, 10.5% respectively). *P. multocida* infection in association with *M. bovis* declared the prevalence of 6.6%.

Molecular investigation of *M. bovis* and *P. multocida* was applied targeting the specific genes of both *M. bovis* and *P. multocida* isolates (*16SrRNA*) detect characteristic bands at (360bp, 460bp respectively). Virulent genes (*VspA* and *toxaA*) showed specific bands at 340bp, 864bp for *M. bovis* and *P. multocida* respectively.

Danofloxacine, and Choloramphenicol were the antibiotics of choice for treatment of both *P. multocida* and *M. bovis* isolates.

Keywords: *M. bovis, P. multocida, PCR, 16SrRNA gene, VspA gene, toxaA gene and antibiotic sensitivity*

Introduction:

Bovine respiratory disease (BRD) is one of the major health problems of cattle worldwide **(Arcangioli *et al*., 2008)**. Bronchopneumonia caused by bovine respiratory disease complex is responsible of economic losses **(Miles, 2009 and USDA, 2013)**. Determining the etiology of bronchopneumonia is a challenge to the veterinarians. Quick and precise diagnostic methods are necessary to prevent bronchopneumonia dissemination in the herd. Animals with respiratory diseases often have depression, anorexia, fever, coughing, increased respiratory and cardiac rates, abnormal respiratory noise and bilateral nasal discharge **(Radostits *et al*., 2007 and Benesi *et al*., 2013)**.

There is an association between respiratory disease and air quality (wet weather and poor

ventilation). Ammonia with dust particles are oftentimes carry microbes, can reach respiratory tissues, whereas they can multiply and cause irritation and inflammatory infections **(Garcia, 2010)**. The cause is usually multifactorial and the disease appears to be as a result of the interaction of infectious microorganisms and such predisposing factors as host defense and environmental stress **(Zakaria *et al*., 2013 & Hartel *et. al*., 2004)**. It is associated with several pathogens, although the majorly reported are *Pasteurella multocida* and *Mycoplasma spp*. **(Holman *et al*., 2015)**, particularly *Mycoplasma bovis* **(Aebi *et al*., 2015).** *M. bovis* increases the severity of respiratory disease in calves and can also act as a primary pathogen **(Gagea *et al*., 2006)**.

The aim of the present study is to throw the light on the prevalence of *M. bovis* and *P. multocida* in cow calves suffered from respiratory manifestations and apparently healthy ones, detection of the antibiotic susceptibility of the isolates and the use of PCR for confirmation of *M. bovis* and *P. multocida* isolates as well as the detection of their virulence genes (*VspA* and *toxaA* genes respectively). Field recommendations should be carried out to protect the animals from respiratory infections.

Material and Methods:

Samples:

A total of 240 samples (nasal swabs, lungs and tracheas) were collected from private farms at Alexandria desert road and slaughter houses from Toukh and El-Quanater abattoirs as well as emergency slaughters at some farms. One hundred eighty (180) samples included in this study were collected from cow calves showing respiratory signs; in-appetence, coughing, sneezing, rapid respiration and nasal discharge as well as 60 samples were collected from apparently healthy animals. Most of the farms subjected to examination had low biosafety level. The total examined samples were 150 nasal swabs, 45 tracheas and 45 lungs collected from living and slaughtered animals as shown in **table (1)**.

Table (1). Samples collected from apparently healthy and diseased cow calves for *Mycoplasma* and *Pasteurella* examination before and after slaughtering

Sample	Apparently healthy calves	Diseased calves	Total
•Nasal swab	30	120	150
*Lung	15	30	45
*Tracheal	15	30	45
Total	60	180	240

•Samples taken before slaughtering *Samples taken after slaughtering

Swab samples were collected using sterile swabs, each trachea and lung samples were kept in a separate sterile plastic bag. All lung samples collected from diseased calves showed pneumonic lesions.

All samples were transferred as soon as possible to the laboratory on ice bag in a cooling container.

Mycoplasma bovis isolation and biochemical characterization:

All samples were cultured by inoculation into PPLO broth media, then plated onto PPLO agar media **(Sabry and Ahmed 1975)** and maintained at 37ºc for 3-7 days with 24-48h observation interval for fried egg colonies.

Biochemical characterization of the isolated purified strains was carried out according to **Watson *et al*., 1988**. Digitonin sensitivity, glucose fermentation, arginine deamination and film & spot formation tests were applied as mentioned by **(Erno and Stipkovits, 1973 & Razin *et al* 1998).**

***Pasteurella multocida* culturing and biochemical identification:**

Nasal swabs were inoculated into casein sucrose yeast (CSY) broth for 6-8 hours, and then a loopful was cultivated onto casein sucrose yeast (CSY) agar, sheep blood agar and MacConkey agar (Oxoid) for bacteriological culturing.

Tissue specimens were cultured directly onto the previously mentioned media. All agar plates were incubated at 37ºC for a minimum of 48 hours. Suspected colonies were picked up for morphological and biochemical identification **(Ruan, 2013, Quinn *et al.*, 2002, & Barrow and Feltman 1993).**

Molecular identification of *M. bovis* and *P. multocida*:

The genomic DNA was extracted from some *M. bovis* and *P. multocida* isolates using Patho Gen-spin DNA / RNA extraction kit (Korea), Cat. No. 17154, following the manufacturers′ recommendations. The extracted DNA samples were subjected to PCR amplification using specific primers and specific profiles as shown in **tables (2, 3)**. The PCR amplified products were electrophoresed through 1.5% agarose gel and DNA was visualized by UV fluorescence after ethidium bromide staining, and then photographed.

All primers were prepared by Sigma Company, Germany.

Table (2). Primers used for molecular identification of *M. bovis* and *P. multocida*

Species	Target gene	Primer sequence (5′→3′)	Amplicon Size(bp)	Reference
M. bovis	*16SrRNA*	CCT TTT AGA TTG GGA TAG CGG TG CCG TCA AGG TAG CAT CAT TTC TAT	360	**Yleana *et al.*, (1995)**
***M.* bovis**	*VspA*	CTT GGA TCA GTG GCT TCA TTA GC GTC ATC ATG CGG AAT TCT TGG GT	340	**Alberto *et al.*, (2006)**
P. multocida	*16SrRNA*	GCT GTA AAC GAA CTC GCC AC ATC CGC TAT TTA CCC AGT GG	460	**Townsend *et al.*, (1998)**
P. multocida	*toxaA*	CTT AGA TGA GCG ACA AGG GAA TGC CAC ACC TCT ATA G	864	**Tang *et al.* (2009)**

PCR reactions were performed in a Gradient Thermal cycler 1000S (Bio – RAD, USA). The reaction mixture (total volume of 50µl) was 25µl Dream green PCR Mix (Dream Taq Green PCR Master Mix (2X) Thermo Scientific Company, Lithuania), 5µl target DNA, 2µl of each primers (containing 10pmole/µl) and the mixture was completed by RNAse DNAse free sterile distilled water to 50µl.

Table (3). PCR amplification for *M. bovis* and *P. multocida* isolates

Thermal profile	*16SrRNA* gene of *M. bovis*	*VspA* gene of *M. bovis*	*16SrRNA* gene of *P. multocida*	*toxaA* gene of *P. multocida*
Initial denaturation		94°C for 5 min	95°C for 4 min	94˚C for 10 min.
Denaturation	94ºC for 45 sec	94°C for 1 min	95°C for 1 min	94˚C for 1 min.
Annealing	60ºC for 1 min	55 °C for 1 min	55°C for 1min	48˚C for 1 min.
Extension	72ºC for 2 min	72 °C for 1.5 min	72°C for 1 min	72˚C for 1 min.
Final extension	72ºC for 3 min	72°C for 10 min	72°C for 9 min	72˚C for 10 min.
Amplification	35 cycles	35 cycles	30 cycles	35 cycles

Antibiotic sensitivity test:

The used antibiotics were commercial ones used in calves' farms. The recovered *M. bovis* isolates were subjected to in-vitro sensitivity test disc and agar diffusion method of **Koneman *et al.* 1997.** The sensitivity of *P. multocida* isolates were carried out using Mueller Hinton agar (Oxoid) plates and the standard disc diffusion method according to **Quinn *et al.* (2002)** using 12 different antibiotic discs. The results were interpreted according to the **CLSI (2017).**

Prevalence and biochemical identification of *M. bovis and P. multocida* in the examined samples:

Pasteurella multocida showed higher incidence than *M. bovis* in samples collected from both apparently healthy and diseased cow calves (6.6 %, 16.6% respectively) (**tables 4, 5**).

The incidence of mixed infection of *M. bovis* and *P. multocida* was 6.6% in diseased cow calves.

All *M. bovis* isolates were digitonin positive. They can′t ferment glucose or hydrolyze arginine but can form film & spot.

Results

Table (4). Prevalence of *M. bovis* and *P. multocida* isolated from apparently healthy cow calves

Samples	No. of examined samples	*M. bovis*		*P. multocida*	
		No.	%	No.	%
Nasal swab	30	2	6.6	4	13.3
Lung	15	0	0	0	0
Trachea	15	1	6.6	0	0
Total	60	3	5	4	6.6

No.= no. of positive isolates
% calculated according to the no. of samples examined

Table (5). Prevalence of *M. bovis* and *P. multocida* isolated from diseased cow calves

Samples	No. of examined samples	*M. bovis*		*P. multocida*		*M. bovis & P. multocida*	
		No.	%	No.	%	No.	%
Nasal swab	120	9	7.5	17	14.16	5	4.1
Lung	30	6	20	8	26.6	3	10
Trachea	30	4	13.3	5	16.6	4	13.3
Total	180	19	10.5	30	16.6	12	6.6

No.= no. of positive isolates
% calculated according to the no. of samples examined

PCR Results:

PCR test was applied on some *Mycoplasma* and *Pasteurella* isolates using the *16SrRNA* specific genes for each of them.

The *16SrRNA* specific gene for *M. bovis* detected a specific band at 360bp. The variable surface protein A (*VspA*) gene showed a characteristic band in some isolates of *M. bovis* at 340bp (**fig. 1, 2**).

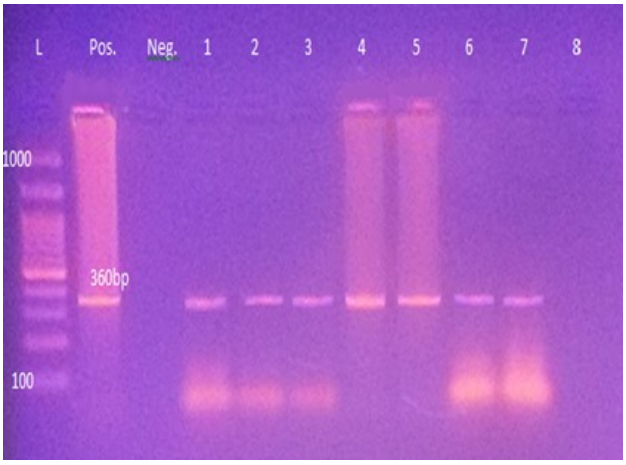

Fig. (1): Agarose gel electrophoretic pattern of *16SrRNA* specific gene of *M. bovis*
Lane 1-7: Positive isolates of *M. bovis* at 360bp
Lane 8: Negative isolate of *M. bovis*

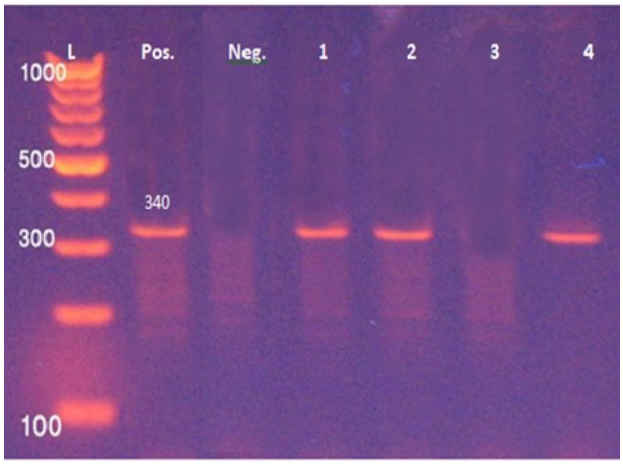

Fig. (2): Agarose gel electrophoretic pattern of *VspA* gene of *M. bovis*
Lane 1, 2, 4: Positive isolates of *VspA* gene of *M. bovis* at 340bp
Lane 3: Negative isolate of *VspA* gene of *M. bovis*

L. = 100bp ladder Pos. = Positive control Neg. = Negative control

The *16SrRNA* specific gene for *P. multocida* showed a characterized band at 460bp and a characteristic band was detected for *toxaA* gene (virulence gene) in some isolates of *P. multocida* at 864bp (**fig. 3, 4**).

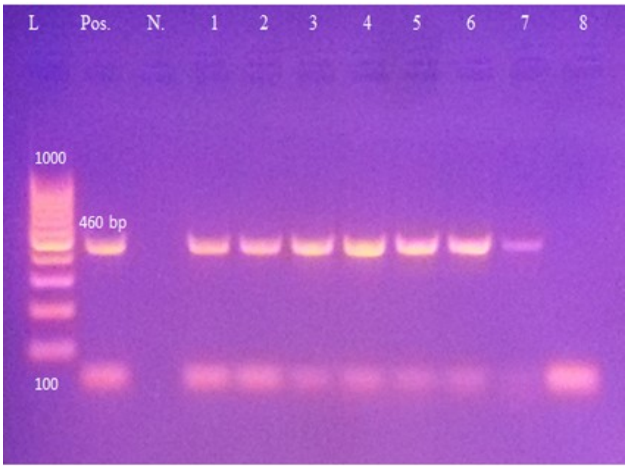

Fig. (3):Agarose gel electrophoretic pattern of *16SrRNA* specific gene of *P. multocida*
Lane 1-7: Positive isolates of *P. multocida* at 460bp
Lane 8: Negative isolate of *P. multocida*

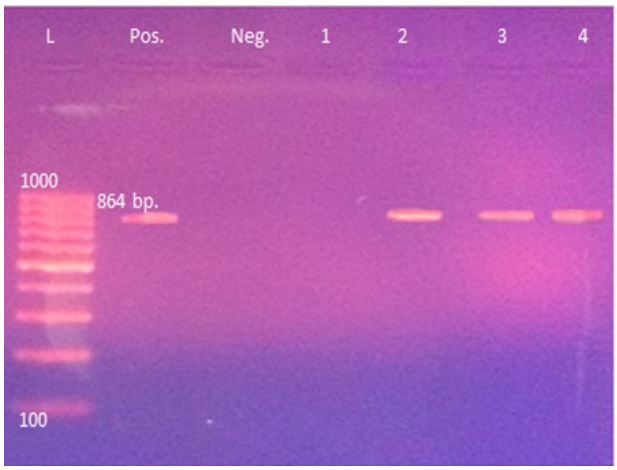

Fig 4: Agarose gel electrophoretic pattern of *toxaA* gene of *P. multocida*
Lane 1: Negative isolate of *toxaA* gene of *P. multocida* at 864bp
Lane 2, 3, 4,: Positive isolates of *toxaA* gene of *P. multocida*

L. = 100bp ladder Pos. = Positive control Neg. = Negative control

Antibiotic sensitivity test results:
Antibiotic sensitivity test was applied on isolated *M. bovis* and *P. multocida* from diseased cow calves. *P. multocida* was sensitive to Danofloxacine, Enrofloxacine, Choloramphenicol and Gentamycine while *M. bovis* showed sensitivity to Choloramphenicol, Danofloxacine, Erythromycin, and Florfenicol as shown in **table (6)**.

Table (6). In-vitro antibiotic sensitivity of *M. bovis* and *P. multocida* recovered from diseased cow calves

Antibiotic disc	Symbol	Potency (µg)	Isolated organism							
			M. bovis (n=19)				*P. multocida* (n=30)			
			Sensitive		Resistant		Sensitive		Resistant	
			No.	%	No.	%	No.	%	No.	%
Choloramphenicol	C	30	18	94.7	1	5.2	27	90	3	10
Ciprofloxacin	CIP	10	7	36.8	12	63.1	9	30	21	70
Danofloxacine	Da	5	16	84.2	3	15.7	28	93.3	2	6.6
Doxycycline	Do	30	7	36.8	12	63.1	26	86.6	4	13.3
Enrofloxacine	ENR	5	8	42.1	11	57.8	28	93.3	2	6.6
Erythromycin	E	15	16	84.2	3	15.7	10	33.3	20	66.6
Florfenicol	FFC	30	15	78.9	4	21.05	17	56.6	13	43.3
Gentamycine	CN	30	11	57.8	8	42.1	26	86.6	4	13.3
Lincomycin	MY	15	9	47.3	10	52.6	11	36.6	19	63.3
Neomycin	N	30	11	57.8	8	42.1	17	56.6	13	43.3
Oxytetracycline	OT	30	13	68.4	6	31.5	7	23.3	23	76.6
Streptomycin	S	25	9	47.3	10	52.6	22	73.3	8	26.6

% calculated according to the no. of tested isolates of *M. bovis* and *P. multocida* (19 and 30 respectively)

Discussion:
Mycoplasmas are one of the pathogens causing bovine respiratory disease. By themselves they can cause a mild respiratory disease, but are more often isolated from pneumonic lungs in association with other pathogens **(Hartel *et al.*, 2004)**. The most common isolates from the upper respiratory tract of the cow calves in our study was *Pasteurella multocida* and *Mycoplasma bovis*.
According to the scientific literature, infection is usually introduced to *Mycoplasma bovis*-free herds by clinically healthy calves or young cattle shedding mycoplasma **(Gourlay *et al.*, 1989)**. Calves can become infected with mycoplasma by drinking milk from cows that have mastitis **(Tenk, 2005)**.
Siugzdaite (2002) carried out investigations in order to detect *M. bovis* from calves aged less than three months of age without clinical symptoms of pneumonia. *M. bovis* was isolated from nasal swabs of 24.4% of calves.
M. bovis and *P. multocida* were present in both diseased and apparently healthy cow calves.
This study was concerned with the molecular investigation of *M. bovis* and *P. multocida* as the main causes of respiratory infections syndrome in cow calves. Thus the recovery rates of *M. bovis* and *P. multocida* were (5%, 10.5%) and (6.6%, 16.6%) respectively from apparently healthy and diseased calves showing respiratory manifestations; coughing, sneezing, rapid respiration; nasal discharge and in-appetence.
These results were less than those reported by **Oliveira *et al.*, 2016** who mentioned that the

prevalence of *Mycoplasma spp*. was 12.5% in which *M. bovis* represents 25% and that of P. multocida was 15.5%.

Our results proved that the incidence of *M. bovis* isolation rate from the lungs was (20%) which were more than those conducted by **Gabinaitiene *et al*., 2011** who revealed that the isolation percentages of *M. bovis* from the lungs of calves were (17.1%). Concerning *P. multocida*, they showed that its incidence in the lungs was 5.7% which was less than our results (26.6%). Also they noticed that the mixed infection of *M. bovis* and *P. multocida* in the nasal swabs was 5.7% which was nearly close to our findings (4.1%), but in lung samples their prevalence was10% in the present study which was higher than those of **Gabinaitiene *et al*., 2011 (**2.9%).

Rasha *et al*., 2014 showed the prevalence of *M. bovis* and *P. multocida* was (8%, 18% respectively) from the nasal swabs which were close to our results (7.5%, 14.16% respectively). Their incidence from the lungs was (6%, 14% respectively) which was less than ours (20%, 26.6% respectively).

Many microorganisms have the ability to vary their size, shape and surface antigens further complicating identification occurred by serological and biochemical assays (**Sachse and Hotzel, 1998**). So the molecular approaches represent a valid and promising option to overcome these limits. The infection is mainly by inhalation of dust mixed with contaminated pulverized droppings. Breeding factors such as animal housing, feeding on moldy hay and ventilation system or environmental factors such as temperature, wind and dew increase the odds of contracting the infection **(Moubasher, 1995).**

Molecular investigation by PCR test was applied to detect the *16SrRNA* and variable surface protein genes (*VspA*) of *M. bovis*. Specific bands at (360bp and 340bp, respectively) were detected for *16SrRNA* and *VspA* genes of *M. bovis* **(Yeleana *et al*., 1995** and **Alberto *et al* 2006)**. Concerning *P. multocida*, the PCR results revealed characteristic bands at (460bp and 864bp, respectively) for its *16SrRNA* and the virulent gene (*toxaA*) proved by **Townsend *et al*, 1998** and **Tang *et al*., 2009**.

Antibiotic sensitivity test results revealed the susceptibility of *M. bovis* to Cholorampheni-col, Danofloxacine, Erythromycin and Florfenicol while *P. multocida* isolates showed susceptibility to Danofloxacine, Enrofloxacine, Choloramphenicol and Gentamycine. Our results agreed with those proved by **Rasha *et al*., 2014.**

Danofloxacine and Choloramphenicol were the best antibiotics for the treatment of both *P. multocida* and *M. bovis*.

Conclusion

Pasteurella multocida and *M. bovis* were the main cause of respiratory infections syndrome in cow calves in farms of bad hygienic conditions and low biosafety measures. The presence of the virulent genes of both *P. multocida* and *M. bovis* plays an important role in the incidence of respiratory infections. Chloramphenicol and Danofloxacin were the antibiotics of choice for the treatment of these respiratory infections.

Recommendations

Strict hygienic measures should be applied in animal farms periodically to get rid of pathogenic agents.

Avoid predisposing factors which initiate and facilitate the respiratory diseases; sudden exposure to air current particularly in closed farms, poor ventilation, overcrowding….etc.

Daily clinical investigation of animals to detect early infection.

Avoid the miss use of antibiotics which cause resistance of the organism to the antibiotics but use those recommended by the laboratory.

Vaccination programs should be strictly applied.

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