Bacteriological Studies On Some Bovine Respiratory System Pathogens Using Recent Techniques

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ABSTRACT

This study was carried out on 556 samples from nasal swabs, lung, bronchial lymph node and trachea, collected from diseased (314) and apparently healthy (242) cattle in different Sharkia and Cairo Governorates. Culturing, identification and serotyping of the isolates showed that the overall prevalence of infection in the examined samples was (65.8%), bacteria were the most prevalent isolates with a percentage of 37.9% followed by Mycoplasma (17%) and Acholeplasma (10.7%). Mixed infection of *Mycoplasma* and bacteria accounted for 20.5%, while mixed infection with different bacterial infection accounted for (8%) in the examined samples.

Polymerase chain reaction (PCR) targeting the 16srRNA gene techniques was used to confirm identification of *M. bovis* by the presence of specific single band at 360 bp and *M. arginini* by the presence of specific single band at 280 bp. Random amplified polymorphic DNA (RAPD) technique showed the genomic diversity among *P. multocida* and *M. haemolytica* field isolates because the banding patterns of field isolates differed from one isolate to another. Mixed infections were estimated to detect the effect of different interactions between microorganisms (Mycoplasma and bacteria) to assist the severity of respiratory disease.

INTRODUCTION

Bovine respiratory disease (BRD) is a major health problem of cattle worldwide. Investigation of microorganisms that affect the respiratory system is very important for evaluation of the role played by different microorganisms in respiratory diseases *(1)*. *Mycoplasma* acts as a stress causing agents leading to a decrease in host defense mechanism and thereby allowing bacteria to invade and colonize the lung causing alveolar pneumonia *(2)*. The bacteria commonly associated with BRD are *Mannheimia haemolytica* type 1A, *Pasteurella multocida* and *Arcanobacterium pyogenes (3)*. Mixed infection of *Mycoplasma* species and other bacteria has been reported *(4)*. PCR technology is the most promising diagnostic technique due to its rapidity, economical convenience and sensitivity *(5)*. The RAPD method has been used to study heterogenicity in closely related organisms *(6)*. The current work was planned for:

1) Isolation and identification of Mycoplasma and bacteria from diseased and apparently healthy cattle in Sharkia and Cairo Governorates, Egypt.
2) Serological identification of the obtained Mycoplasma isolates using standard antisera.
3) molecular identification of mycoplasma isolates Using PCR technique to confirm the Mycoplasma isolated strains.
4) Using random amplified polymorphic DNA (RAPD) technique to differentiate between *M. haemolytica* and *Pasteurella multocida* isolates.

MATERIAL AND METHODS

Samples: Five hundred and fifty six samples from nasal swabs, lung, bronchial lymph nodes and tracheal samples were collected from diseased and apparently healthy cattle from Sharkia (Facous) and Cairo (El-Basateen) Governorates. The samples were studied bacteriologically.

Isolation of Mycoplasma: The culture media were heart infusion broth and heart infusion agar media *(7)*.

Isolation of bacteria: The culture media were nutrient broth, nutrient agar, blood agar, DAS, MacConkey bile salt, lactose agar, mannitol salt agar, Baired parker, eosin methylene blue, modified Edwards and semisolid 0.5% agar media *(8)*.

Genus determination of Mycoplasma and Acholeplasma by using digitonin test *(9)*.

Biochemical characterization: glucose fermentation test *(10)*, arginine deamination test *(10)* and film and spot formation test *(11)* were carried out. The isolated Mycoplasma were classified into four groups (104) according to the biochemical tests (Table 4).

Biochemical tests of bacteria *(12)*: Indole test, methyl red, voges prauskauer reaction, citrate utilization test, urease test, gelatin liquification test, aesculin hydrolysis test, sugar fermentation test and nitrate reduction test were carried out.

Serotyping of Mycoplasma isolates: growth inhibition test *(13)* was applied. The reference antisera (Animal Health Research Institute Dokki, Giza) were kindly supplied by Mycoplasma Department of Animal Health Research Institute, Egypt.

PCR and RAPD were carried out as follow *(6)*:

DNA extraction.

DNA quantification

DNA amplification: the primers used for detection of *M. bovis (14)* were:

Forward: 5'- CCT TTT AGA TTG GGA TAG CGG ATG -3`.

Reverse: 5'- CCG TCA AGG TAG CAT CAT TTC CTA T -3`.

The primers used for detection of *M. arginini (15)* were:

Forward: 5'- GGG AGC AAA CAG GAT TAG ATA CCC T -3'.

Reverse: 5'-TGC ACC ATC TGT CAC TCT GTT AAC CTC -3'.

After adding all reagents (oligonucleotide primers, 10xTaq Buffer, 10 mM dNTPs {deoxynucleotides tri phosphate solution}, Taq thermostable DNA polymerase, $MgCl_2$ {magnesium chloride}, DNase- RNase- free deionized water, Template DNA) for DNA amplification, the reaction was carried on the progene thermal cycler.

Identification of the PCR products by electrophoresis

The following random primers were used for RAPD-PCR in detection of *P. multocida (6)* and *M. haemolytica (16)*.

OPA-O2 random primer with sequence TGCCGAGCTG

OPA-20 random primer with sequence GTTGCGATCC

OPA-O1 random primer with sequence GTTTCGCTC

OPB-O4 random primer with sequence GGACTGGAGT

OPB-O8 random primer with sequence GTCCACACGG

RESULTS

The total prevalence of positive samples was higher in diseased cattle (73.5%) than from apparently healthy (55.7%) as shown in Table 1.

The results from Tables 2 and 3 revealed that the highest prevalence of positive samples was from bacteria (37.9%) followed by Mycoplasma (17%), while mixed infection representing 28.8%, distributed as mixed bacteria + Mycoplasma (20.5%) and mixed bacterial infection (8.2%).

After applying biochemical identification and serotyping of Mycoplasma, it was clear from the results in Table 4 that *M. arginini* was the highest Mycoplasma species, isolated with a percentage of 7.2%, while the prevalence of *M. bovis* was 6.8%, *M. bovirhinis* 1.6% and *M. bovigenitalium* 1.4%.

The isolated Mycoplasma distribution in diseased animals was *M. arginine* (29 positive isolates) followed by *M. bovis* (27 positive) while from apparently healthy animals the *M. arginine* and *M. bovis* were equally distributed as 11 positive isolates. *M. arginine* more prevalent in lung of diseased and apparently healthy animals distributed as 11 and 5 isolates.

M. bovirhinis was more prevalent in nasal swabs of diseased cattle (4 isolates), failed to be isolated from lung and bronchial lymph nodes of apparently healthy cattle. *M. bovis* was more prevalent in nasal swabs of diseased cattle (10 isolates) followed by lung (7 isolates). In apparently healthy animals the lowest incidence was from tracheal samples (one isolate).

M. bovigenitalium was more prominent in nasal swabs of diseased animals (3 isolates) and failed to be isolated from bronchial lymph nodes of both diseased and apparently healthy cattle as illustrated in Table 5.

Biochemical identification of bacteria revealed from Table 5 showed that *P. multocida* was the highest prevalence organism (6.44%) while the lowest was from *Salmonella* species (2%) as shown in Table 6.

The total prevalence of mixed infection in collected samples was 17% (Table 7). *P. multocida* + *M. bovis* + *Staph.* species were the highest prevalence (3.2%), while the lowest incidence was for *M. bovigenitalium* + *Staph.* species and *M. bovirhinis* + *P. multocida* + *E. coli* was 1% each.

PCR technique confirmed the result of *M. bovis* by the presence of specific single band at 360 bp (figure 1) and *M. arginini* by the presence of the specific single band at 280 bp (figure 2).

RAPD technique showed the intra-species heterogenecity among 2 strains of *Pasteurella multocida* and one from *M. haemolytica* field isolates with the reference strain (figure 3 and 4) and analyzed in Tables 8 and 9. *P. multocida* (isolate 1) was isolated from nasal swabs while *P. multocida* (isolate 2) was isolated from lung. *M. haemolytica* (isolate 3) was isolated from nasal swabs.

Table 1. The incidence of positive samples from diseased and apparently healthy animals.

Sample	Diseased animals			Apparently healthy animals			Total		
	No. of samples	Positive samples	%*	No. of samples	Positive samples	%**	No. of samples	Positive samples	%
Nasal swabs	90	77	24.5	76	53	21.9	166	130	78
Tracheal	70	41	13	60	36	14.9	130	77	59
Lung	74	64	20	56	39	16	130	103	79
Bronchial lymph nodes	80	49	15.6	50	7	2.8	130	56	43
Total	314	231	73.5	242	135	55	556	366	65.8

* according to total diseased (314) ** according to total apparently healthy (242)

Table 2. Total prevalence of Mycoplasma and bacteria of positive samples recovered from diseased and apparently healthy animals

	Mycoplasma		Bacteria		Total	
	No.	%	No.	%	No	%
Nasal swabs (166)	35	21	77	46	112	67
Tracheal samples (130)	15	11.5	45	34.6	60	46
Lung samples (130)	32	24.6	55	42	87	66.9
Bronchial lymph nodes (130)	13	10	34	26	47	36
Total	95	17	211	37.9	306	55

Table 3.Total prevalence of mixed infection of positive samples recovered from diseased and apparently healthy animals

Types of infection	Sites									
	Nasal swabs (166)		Tracheal samples (130)		Lung (130)		Lymph nodes (130)		Total (556)	
	No. of +ve	%	No. of +ve	%	No. of +ve	%	No. of +ve	%	No. of +ve	%
Mixed bacterial infection	18	10.8	9	6.9	15	11.5	4	3	46	8.2
Bacteria and *Mycoplasma*	40	24	23	17.6	46	35	5	3.8	114	20.5
Total	58	10.4	32	5.7	61	10.9	9	1.6	160	28.8

Table 4. Summary of biochemical characterization groups of the isolated *Mycoplasma*

Biochemical groups	Suspected species	Isolates	
		No. of +ve samples	%*
Group 1 Glucose negative Arginine positive Film and spot negative	*M. arginine*	(40)	7.2
Group 2 Glucose positive Arginine positive Film and spot negative	*M. bovirhinis*	(9)	1.6
Group 3 Glucose negative Arginine negative Film and spot positive	*M. bovigenitalium* *M. bovis*	(8) (38)	1.4 6.8
Total		(95)	17

* Calculated number of positive *Mycoplasma* 95/total number of examined samples (556).

Table 5. Frequency distribution of biochemical groups of the isolated Mycoplasma

Samples	Diseased				Apparently healthy				Total
	M. arginine	*M. bovirhinis.*	*M. bovis*	*M. bovigenitalium*	*M. arginine*	*M. bovirhinis.*	*M. bovis*	*M. bovigenitalium*	
Nasal swabs	9	4	10	3	3	1	4	1	35
Tracheal	6	0	4	1	2	1	1	0	15
Lung	11	2	7	1	5	0	4	2	32
Bronchial lymph nodes	3	1	6	0	1	0	2	0	13
Total	29	7	27	5	11	2	11	3	95

Table 6. Prevalence of bacterial single infection recovered from cattle respiratory system

Types of isolated bacteria	No. of isolates	Total no. of samples	%
P. multocida	36	556	6.4
M. haemolytica	31		5.5
E. coli	26		4.6
Staphylococcus spp.	22		3.9
Streptococcus spp.	21		3.7
Klebsiella spp.	17		3
Salmonella spp.	12		2
Proteus spp.	15		2.6
Pseudomonas spp.	17		3
Corynebacterium spp.	14		2.5
Total	211		37.9

*: Percentage of total number of positive single bacterial infection/total number of examined samples (556).

Table 7. Total prevalence of mixed infection in collected samples (556)

Identified microorganism	Diseased		Apparent healthy		Total	
	No.	%•	No.	%*	No.	%*
E. coli + *P. multocida*	10	3	1	0.4	11	1.9
P. multocida + *Strept.* spp.	8	2.5	2	0.8	10	1.7
E. coli +*Klebsiella* spp.	6	1.9	2	0.8	8	1.4
Staph. spp. + *P. multocida*	6	1.9	1	0.4	7	1.2
M. arginine + *M. haemolytica* + *Proteus* spp.	10	3	5	2	15	2.6
P. multocida + *M. bovis* + *Staph.* spp.	16	5	2	0.4	18	3.2
M. bovigenitalium + *P. multocida* + *Strept.* spp.	9	2.8	1	0.8	10	1.7
M. bovigenitalium + *E. coli* + *Strept.* spp.	6	1.9	2	0.8	8	1.4
M. bovirhinis + *P. multocida* + *E. coli*	4	1	2	0.8	6	1
M. bovigenitalium + *Staph.* spp.	2	0.6	1	0.4	3	1
Total	77	24.5	19	7.8	96	17%

*: Percentage of total number of mixed infection isolates/total number of examined samples.

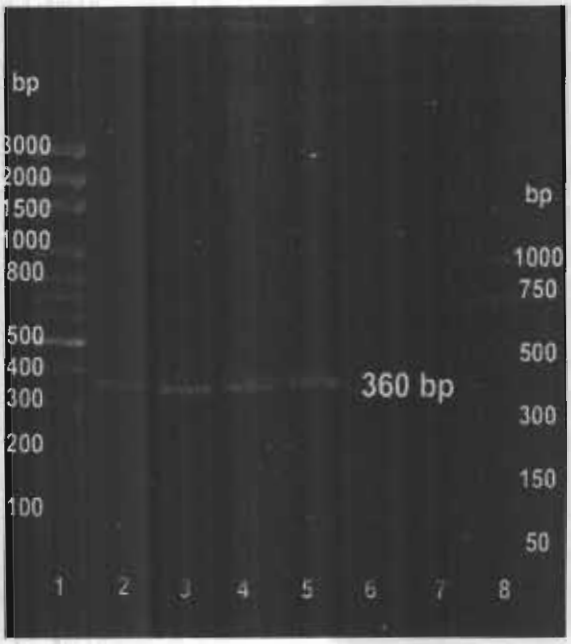

Figure 1. Agarose gel electrophoresis of *M. bovis* DNA (PCR). Lane 1: Ladder (Axgen biosciences). Lane 2-7: *M. bovis* field isolates, (8). Control (Promega).

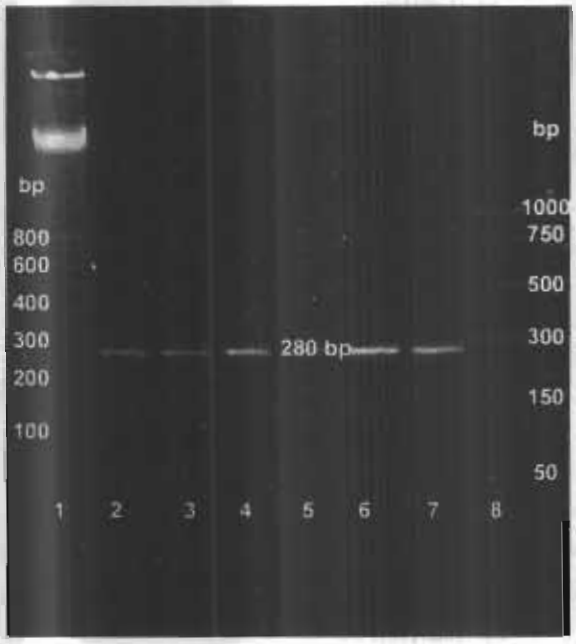

Figure 2.Agarose gel electrophoresis of *M. arginini* DNA (PCR). Lane 1: Ladder (Pharmacia). Lane 2-7: *M. arginini* field isolates, (8). Control (Promega).

Figure 3. Agarose gel electrophoresis of RAPD-PCR products of isolates using OPB-04 random primer. Lane 1: ladder, Lane 2, 3 *P. multocida* field isolates, Lane 4: *M. haemolytica* field isolates, Lane 5: Control ladder.

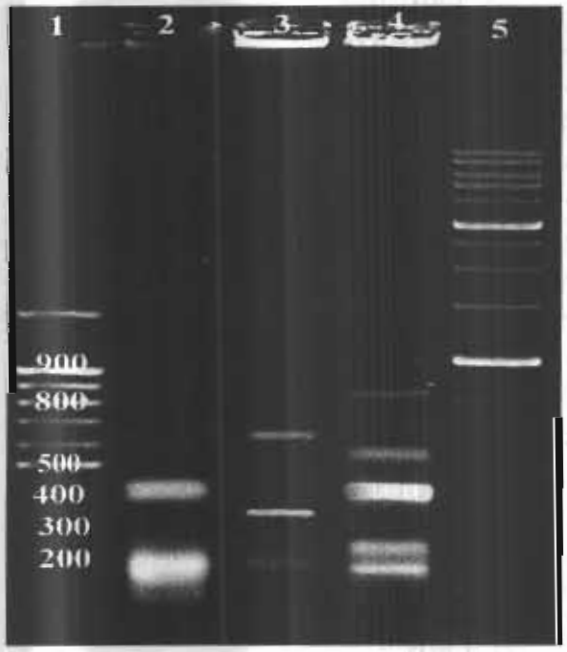

Figure 4. Agarose gel electrophoresis of RAPD-PCR products of isolates using OPB-08 random primer. Lane 1: ladder, Lane 2, 3 *P. multocida* field isolates, Lane 4: *M. haemolytica* field isolates, Lane 5: Control ladder.

Table 8. Polymorphic bands detected among *P. multocida and M. haemolytica* isolates using OPB-04 random primer

Molecular weight of polymorphic bands (bp)	Isolate 1	Isolate 2	Isolate 3
945	-	+	-
930	+	-	+
738	-	+	-
542	-	-	+
536	+	-	-
430	-	+	-
320	+	-	+
295	-	+	-
210	+	-	+

Table 9. Polymorphic bands detected among *P. multocida and M. haemolytica* isolates using OPB-08 random primer

Molecular weight of polymorphic bands (bp)	Isolate 1	Isolate 2	Isolate 3
850	-	-	+
630	-	+	-
552	-	-	+
410	+	-	-
400	-	-	+
342	-	+	-
254	-	-	+
226	-	+	-
218	+	-	-
211	-	-	+

DISCUSSION

Respiratory infection is a complex syndrome and its etiology involves many different factors including stress factors, environmental factors, bacteria, mycoplasmal and viral infections *(17)*. There are several *Mycoplasma* species colonizing the bovine respiratory mucous membranes; *Pasteurella* and *Mannheimia* species can act as primary or secondary opportunistic pathogens causing bovine pneumonia *(18)*.

The total prevalence of positive samples (Tables 1 and 2) showed that the highest prevalence was in diseased cattle compared with apparently healthy animals. Previous studies showed similar prevalence *(17, 19)*.

Biochemical and serotyping of the *Mycoplasma* revealed different types of *Mycoplasma* species involving *M. arginini*, *M. bovirhinis*, *M. bovis* and *M. bovigenitalium* (Table 4). These results coincide with another study where *Mycoplasma arginini* was isolated as 18 strains, *Mycoplasma bovigenitalium* (10 strains) *(21)*. *M. bovis* was the highly isolated *Mycoplasma* species in avian species *(6)*.

M. arginini was more prevalent from lung samples (11 isolates) in diseased animals than from the apparently healthy. *M. bovirhinis* was isolated from nasal swabs of diseased animals, while failed to be isolated from lung and

bronchial lymph nodes of apparently healthy cattle. *M. bovis* was highly prevalent from nasal swabs of diseased cattle, while was the lowest from tracheal samples of apparently healthy cattle. *M. bovigenitalium* its highest incidence was from nasal swabs of diseased animals while failed to be isolated from both tracheal and bronchial lymph nodes of apparently healthy animals.

M. bovis was recovered from 203 nasal swabs of 11 healthy and 14 diseased animals, 43 from 245 tracheal swabs. While *M.* arginine was recovered from lungs (10 strains). *M. bovigenitalium* was isolated from pneumonic lungs (5 strains). While *M. bovirhinis* failed to be isolated *(29)*.

The isolation of bacteria was 37.9%, Mycoplasma 17%, mixed Mycoplasma + bacteria 20.5% and mixed bacterial infection 8.2% (Table 3).Recently several studies showed similar prevalence of Mycoplasma and bacterial infection from cases of mastitis in cows and buffaloes *(19)* and feedlot buffalo calves suffering from pneumonia *(20)*.

Similar to several previously reported studies *(20, 22-24)*. This study showed that biochemical identification of bacteria indicated that *P. multocida* was the highest prevalent isolate 6.4% while the lowest incidence was *Salmonella* species (Table 5).

The total prevalence of mixed infection in the examined samples was 17%. *P. multocida* + *M. bovis* + *Staphylococcus* species were the highest. *M. bovigenitalium* + *Staph* species and *M. bovirhinis* + *P. multocida* + *E. coli* were the lowest. Isolation of M. bovis from bovine clinical cases in Ireland *(25, 26)* and bacterial mastitis in cows and buffaloes *(19)* revealed similar prevalence in Ireland.

PCR technique confirmed *M. bovis* (figure1) in six field isolates by the presence of M. bovis which showed specific single band at 360 bp *(14, 15, 17)*. *M. arginini* was detected in six field isolates by the presence of specific single band at 280 bp *(5)*.

Intra-species heterogenicity among field isolates of *P. multocida* and *M. haemolytica* isolated from cattle have been studied by RAPD which showed the different banding pattern of each field isolates of *P. multocida* (Table 7 and figure3) and for *M. haemolytica* (Table 8 and figure 4) and it proofs that the extra DNA-sequence occurred in each individual strain is due to the isolation from different localities and this can help in epidemiological studies of such microorganism if an outbreak occurred. Each isolate had many amplified bands in common, but strain to strain variation could be detected by the presence or absence of some other bands *(16 , 27)*.

The obtained results illustrated the role played by respiratory bacterial pathogens and Mycoplasma in complication and severity of the respiratory disease. So, proper diagnostic identification will help in prevention and treatment of the causative agents.

No doubt that the use of PCR technique can overcome the disadvantages of culturing and serological methods in both the time needed and the accurateness of the results. PCR is the most sensitive, specific test that can be used instead of prolonged culturing and serotyping of the isolates *(28)*.

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الملخص العربى

دراسات بكتريولوجية على بعض ممرضات الجهاز التنفسى فى الأبقار بإستخدام التقنيات الحديثة

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قسم الميكوبلازما – معهد بحوث صحة الحيوان – الدقى – جيزة

هذه الدراسة أجريت على ٥٥٦ عينة عبارة عن مسحات أنفية ، رئة ، غدد ليمفاوية والقصبة الهوائية ووزعت كالآتى: ٣١٤ عينة من الحيوانات المريضة ، ٢٤٢ عينة من الأبقار السليمة ظاهرياً من مختلف محافظات الشرقية والقاهرة. بعد الزرع والتصنيف السيرولوجى للعزلات قد تبين أن البكتريا كانت أكثر المعزولات بنسبة حدوث (٧٥،١٪) يتبعها الميكوبلازما (٤٣٪) والأكوليبلازما (غير الممرضة) (٢١،٥٪) بنسبة حدوث (٦٥،٧٪) ثم العدوى المختلطة من الميكوبلازما والبكتريا ، والعدوى المختلطة من البكتريا المتنوعة بنسب ٢٠،٥٪ ، ٨٪ على التوالى.

تقنيات تفاعل البلمرة المتسلسل للحامض النووى ريبو لتأكيد تصنيف الميكوبلازما أرجينينى بوجود باند مفردة خاصة عند ٢٨٠ قاعدة مضاعفة وميكروب الميكوبلازما بوفس وجود باند مفردة عند ٣٦٠ قاعدة مضاعفة وكذلك تقنية التكبير العشوائى المتعدد للحامض النووى أظهرت التعدد الجينى بين الباستريلا مالتوسيدا والمانهيميا هيموليتيكا كأنماط للمعزولات الحقلية والتى اختلفت من عزلة لأخرى. والعدوى المختلطة استخدمت لتقييم تأثير مختلف التفاعلات التى قد تحدث بين مختلف الميكروبات والضغط والبيئة المحيطة بالحيوان.