Study On Some Mycological, Mycoplasmal And Bacteriological Causes Of Pneumonia In Cattle

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ABSTRACT

Bovine respiratory disease (BRD) caused by various Mycoplasma, other bacteria or fungi is a major health problem of cattle worldwide. The present study was conducted to examine 100 nasal swabs of cattle (1-5 years) reared in private farms at El-Menoufea governorate showing Symptoms of depression , reduced feed intake, respiratory problems, dyspnea with or without nasal discharge and pneumonia. 50 lung tissues collected from different abattoirs at El- Menoufia Governorate and obtained from part showing pneumonia.

Mycoplasma bovis, *Pasteurella multocida* and *Mannhiema haemolytica* were isolated in percentages of (8%, 18% and 12%) and (6%, 14% and 10%) in both nasal swabs and lung tissues respectively. The main isolated moulds and yeasts, were *Asperigullus fumigatus*, *Asperigullus niger*, *Asperigullus candidus*, *Asperigullus flavus*, *Penicillum sp.* and *Candida sp.* which were isolated in percentages (16% , 72% , 16%, 8% , 32% and48%) and (8% , 8% , 0% ,8% , 24% and 40%) in both nasal swabs and lung tissues respectively. Polymerase chain reaction (PCR) is very specific, simple, sensitive and rapid diagnosis of *Mycoplasma bovis*, *Pasteurella multocida*, *Asperigullus fumigatus*, *A Asperigullus niger*, *Asperigullus flavus* and *Candida species. Pasteurella sp.* isolates were sensitive to Tulthromycin and Gentamicine antimicrobials while *M. haemolytica* isolates were sensitive to Tulthromycin (Draxxin) Nevertheless, *Mycoplasma bovis* isolates were sensitive to Tulthromycin (Draxxin) and Ciprofloxacin.

INTRODUCTION

Bovine respiratory disease (BRD) is a major health problem of cattle worldwide ***(1)***. It inflicts considerable economic losses in beef herds ***(2,3)***. The cause is multifactorial and disease appears to result from the interaction of infectious microorganisms and such predisposing factors as host defense, environmental stress ***(4)***. *Mycoplasma bovis* is a common inhabitant of the upper and lower respiratory tract of healthy cattle. This *Mycoplasma sp.* increases the severity of respiratory disease in calves and can also act as a primary pathogen ***(5)***.

Pasteurella multocida, *Mannhiema hemolytic, Histophilus somni*, *Mycoplasma bovis* and *Mycoplasma dispar* are usually associated with concurrent virus infection ***(1)***. There is an association between respiratory disease and air quality (wet weather and poor ventilation) in confinement environments as raising calves in barns in which warm air, ammonia, dust and microorganisms (e.g. fungal spores, viruses and bacteria). Ammonia with dust particles which often times carry microbes, can reach respiratory tissues, whereas they can multiply and cause irritation and inflammatory infections ***(6)***. Mycotic infection is mainly caused by inhalation of spores, which can lead to haemo-lymphatic dissemination. *Aspergillus species*, *Cryptococcus neoformans* and *Candida species*, are identified as the main causative agents of mycotic pneumonia ***(7)***. They cause significant economic losses, morbidity and mortality in animals and immunological

compromised humans, where it is capable of killing cells by causing extensive damage to cellular membrane *(8 ,9)*.

The severe pneumonic damage characterized by pulmonary invasion of *Pasteurella multocida* and *Mannheim haemolytica* and other bacteria is associated with the production of virulence factors which facilitate colonization of the lower respiratory tract *(10)*. Therefore the present study aimed to investigate the main mycoplasmal, bacterial and fungal causes of bovine respiratory disease (BRD) and to develop a simple, sensitive, specific and rapid diagnosis of the microbiological agents in Egyptian cattle by using Polymerase chain reaction (PCR.)

MATERIALS AND METHODS

Samples: A total of 100 nasal swabs were collected aseptically from cattle (1–5 years) reared in private farm at El- Menoufea governorate showing respiratory manifestation. Samples were collected by means of sterile cotton swabs and transported to the lab. as quickly as possible in sterile peptone water 1% for bacteriological examination. Also 50 pneumonic lung tissues samples were collected from different abattoirs at El- Menoufea Governorate in sterile bags and transported to the lab, quickly as possible.

Isolation of Mycoplasma sp.: Samples were cultured by inoculation in broth media, then plating onto PPLO agar media *(11)* and maintained at 37ºC for 3-7 days. Genus determination *(12)*, Biochemical characterization of the isolated purified strains was carried out, *(13)*. Film and spot formation *(14)*. The isolates were serologically identified by growth inhibition *(15)*.

Isolation and identification of *Pasterulla sp.*: The collected samples were inoculated onto blood agar and Macconkey agar which were incubated aerobically at 37 ºC for 24 – 48 hrs. Suspected growing colonies onto the surface of above mentioned media were identified on the basis of their colonial morphology and staining reaction. Purified colonies showed typical characters of *Pasterulla sp.* were picked up and streaked into semi – solid agar media and incubated at 37 ºC for 24 hr. The pure colonies were biochemically identified according to *(16)*.

Mycological isolation : This was carried out according to *(17)*. Sabouraud's dextrose agar (DIFCO) containing 0.05 mg/ml chloramphenicol (to inhibit bacterial growth) was prepared.

Identification of isolated moulds : The inoculated plates were incubated at 25 ºC / 7 days and examined daily.

Identification of isolated moulds was based on their growth rate and colonial morphology. Then it was confirmed microscopically.

Identification of yeasts :The inoculated plates were incubated at 37 ºC / 24-48hr, the morphology and staining reaction of isolates were observed after staining by the Indian ink stain and Gram's stain. Pure yeast colonies grown on SDA were subcultured on Rice agar medium with polysorbate 80 then examined microscopically after 48 h at 25ºC for identification and characterization of *candida species*.

Antibiogram technique: All isolates identified as *Mannhiema haemolytica* and *Pasteurella multocida* were subcultured onto Muller-Hinton agar, *Mycoplasma bovis* were subcultured onto PPLO agar for antimicrobial sensitivity test to detect the drug choice according to *(18)*. These antimicrobial were including Tulthromycin, Ciprofloxacin Oxytetracycline, Amoxicllin, Gentamicin, Enrofloxacin, Florfenicol, Trimethoprim-sulphamethosazole and Cephaloxin .

Polymerase chain reaction (PCR): Application of PCR technique for identification of *Mycoplasma bovis P. multocida A. fumigatus A. flavus A. niger* and *Candida sp.* isolates were performed essentially by using primers (Chromogen Company, South Korea) as shown in Table (1).

Table 1. Primers Name, Sequence and Amplicon size of primers used

Sp.	Primer pairs	Sequence (5′→3′)	Amplicon size (bp)
M.bovis	F	ACACCATGGGAGYTGGTAAT	350-490
	R	CTCCWTCGACTTYCAGACCCAAGGCTA	
P. multicodia	KMT1SP6	GCTGTAAACGAACTCGCCAC	460
	KMT1T7	ATCCGCTATTTACCCAGTGG	
A. fumigatus	PEX1	TATGTCTTCCCCTGCTCC	250
	PEX2	CTATGCCTGAGGGGCGAA	
A. flavus	PEPO1	CGACGTCTACAAGCCTTCTGGAAA	200
	PEPO2	CAGCAGACCGTCATTGTTCTTGTC	
A. niger	PEPI1	CCAGTACGTGGTCTTCAACTC	150
	PEPI2	CTATTGTACCTTGTTGCTTCGGCG	
Candida spp	ITS3	GCATCGATGAAGAACGCAGC	400
	ITS4	TCCTCCGCTTATTGATATGC	

DNA extraction of Mycoplasma bovis *(19)*.5ml of a 24 hour broth cultures of isolates were centrifuged for 10 minutes at 12000 r.p.m. The pellet was washed twice in 200 µl of PBS pH 7.2 and suspended in 25 µl PBS. The cell suspension was heated directly at 100°C for 10 min. in a heat block to break the cell membranes, and then cooled on ice for 5 min. Finally, the cell suspension was centrifuged for 5 min. and the supernatant containing DNA was collected and stored at -20°C until used.

DNA Extraction Method of *P. multocida*

To extract DNA of *P. multocida* we used *(20)* method since the broth culture of *P. multocida* was transferred to an Eppendorf tube and centrifuged at 3000-x g for about 10 minutes. The pellet obtained after centrifugation was washed and resuspended in PBS and then centrifuged again. The final pellet was resuspended in 100 µl of distilled water. The mixture was boiled for 10 minutes in water bath and transferred immediately into ice and snap chilled for 30 minutes. The sample was then thawed and centrifuged at 3000 x g for 5 minutes. The supernatant was separated from pellet and used as template DNA.

DNA extraction of *Aspergillus sp.* and *Candida sp.*

To extract *Aspergillus* sp., candida *sp.* DNA, we used (QIAamp DNA mini kit; Qiagen, Hilden, Germany, cat. No.69104) Extraction Kit with following the manufacturer's instructions.

PCR amplification

Amplification of *Mycoplasma bovis (21)* was as follow: Pre-PCR step for 10 minute at 95°C, then 35 cycles of denaturation for 30 sec at 94°C, annealing for 45 sec. at 60°C, and extension for 1 min at 72°C. Amplification conditions for *A. fumigatus, A. flavus and A. niger* were: 5 min initial step followed by 38 cycles at 94 °C for 1 min, 59 °C for 1 min and 72 °C for 1.5 min and a final extension step at 72 °C for 5 min. The PCR amplification protocol used for Candida was as follows: 1 cycle of 4 min 30 s at 95°C, 30 cycles of 30sat95°C (denaturalization), 30 s at 63°C (annealing), 1 min at 72°C (extension) and finally 1 cycle of 3 min at 72°C. Amplification condition of *Pasteurella multocida (22)* was the initial denaturation at 94°C for 5 minutes; 30 cycles of 94˚C for 1 minutes, 53°C for 1 minutes, and 72˚C for 1 minute; and final extension at 72°C for 9 minutes.

PCR reactions were performed in an Gradient Thermal cycler (1000 S Thermal cycler Bio-RAD USA).The reaction mixture (total volume of 50 µl) was 25 µl Dream green PCR Mix (Dream Taq Green PCR Master Mix (2X) Fermentas Company, cat., no.K1080, USA.), 5 µl target DNA, 2 µl of each primers

(containing 10 p mole/ µl) and the mixture was completed by sterile D. W. to 50 µl. The amplified products were analyzed by electrophoresis on 1.5 % agarose (Agarose, Sigma ,USA) gel after ethidium bromide staining . After polymerization of the gel, samples were added to fill the wells dip and using GeneRuler 100bp DNA Ladder: Fermentas Company, Cat.no. SM0243,US. . The gel tank was filled with 1X Tris Boric EDTA (TBE) buffer up to the wells dip and run at a constant current of 80 Volts. The final gel was viewed by UV transillumination then photographed.

RESULTS

Isolation and Molecular identification: In the present study, results were summarized in Table (2) which shows that in nasal swab the prevalence of *M. bovis* was 8%, *P . multocida* (18%), *M . haemolytica* (12%), *A . flavus* (16%), *A . niger* (72%), *A . candidus* (16%), *A . fumigatus* (8%), *Penicillium sp* (32%), and *Candida sp* (56%) while in Lung tissue The prevalence of *M . bovis* (6%). *P . multocida* (14%), *M . haemolytica* (10%), *A . flavus* (8%), *A . niger* (8%), *A . fumigatus* (8%), *Penicillium sp.* (24%) and *Candida sp.* (40%).

Table 2. Prevalence of *Mycoplasma bovis*, *Pasteurella multocide*, *Mannheima haemolytica*, *Aspergillus sp. and Candida sp.* isolated from nasal swabs and lung tissues of cattle suffering from respiratory manifestation

Samples / Species	Nasal swabs (100)		Lung tissue (50)	
	Positive No.	%	Positive No.	%
M . bovis	8	8	3	6
P . multocida	18	18	7	14
M . haemolytica	12	12	5	10
A . flavus	16	16	4	8
A . niger	72	72	4	8
A . candidus	16	16	-	-
A . fumigatus	8	8	4	8
Penicillium sp.	32	32	12	24
Candida sp.	56	56	20	40

We subjected DNA of the isolates to specific PCR to *M. bovis, P . multocida, A . fumigatus , A . niger , A . flavus* and *Candida sp.* Fig (1-6).

Sensitivity test results Table (3): shows the antibiogram of the isolates, it was clear that all the isolates of *P . multocida* were sensitive to Tulthromycin, and Gentamicin *M . haemolytica* isolates were more sensitive to Tulthromycin Nevertheless *M. bovis* isolates were more sensitive to Tulthromycin and Ciprofloxacin.

Table 3.Antibiogram results of the isolates

Isolates / Antibiotic	*P. multocida*	*M. haemolytica*	*Mycoplasma bovis*
Tulthromycin	+++	+++	+++
Ciprofloxacin	++	+	+++
Oxytetracycline	+	-	+
Amoxicllin	+	+	-
Gentamicin	+++	++	+
Enrofloxacin	++	++	+
Florfenicol	+++	++	-
Sulfamethosazoale	+	+	-

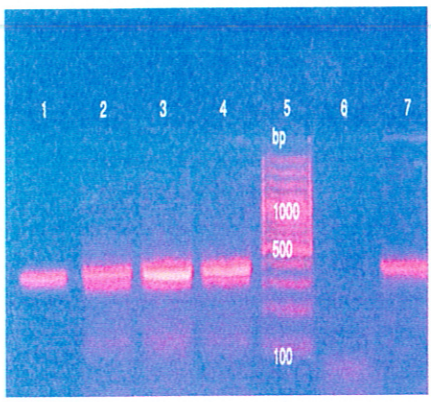

Fig 1. Agarose electrophoretic pattern of M. bovis
Lane 1-4 : positive samples
Lane 5 : 100 bp DNA Ladder
Lane 6: control Negative
Lane 7: Control positive

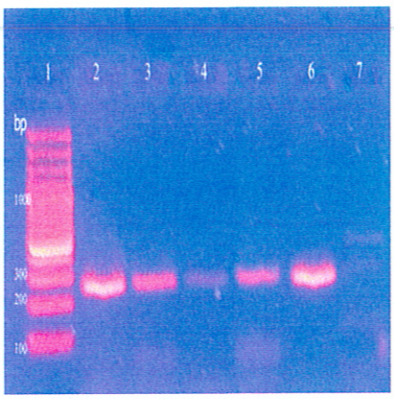

Fig2. Agarose electrophoretic pattern of *A. fumigatus*
Lane 1: 100 bp DNA ladder
Lane2-5:positive isolates to *A.fumigatus*
Lane 6: control positive
Lane 7: control Negative

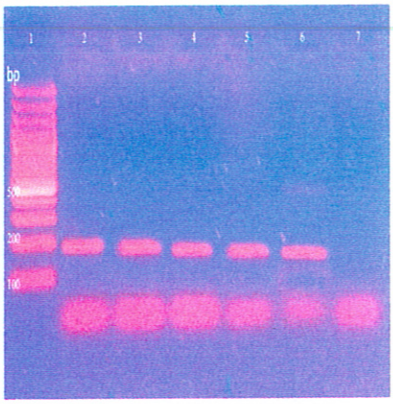

Fig 3. Agarose electrophoretic pattern of *A. flavus*
Lane 1: 100 bp DNA ladder
Lane2-5:positive isolates to *A.flavus*
Lane 6: control positive
Lane 7: control Negative

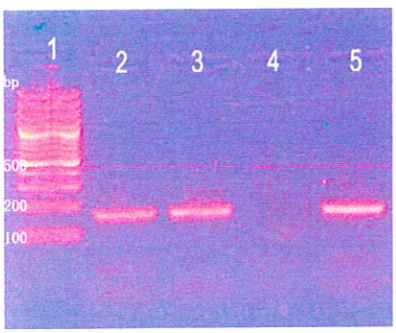

Fig4: Agarose electrophoretic pattern of *A. niger*
Lane 1: 100 bp DNA ladder
Lane 2,3: positive isolates to *A.niger*
Lane 4: control negative
Lane 5: control positive

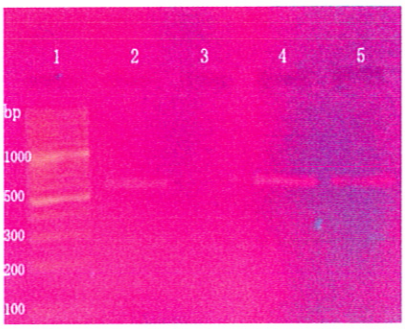

Fig 5: Agarose electrophoretic pattern of *Candida sp.*
Lane 1: 100 bp DNA ladder
Lane 2:control positive
Lane 3: control negative
Lane4, 5: positive isolates to *Candida sp.*

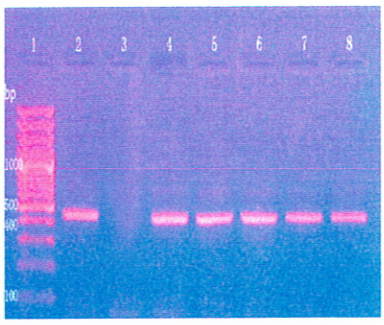

Fig 6: Agarose electrophoretic pattern of *P. multocida*
Lane 1: 100 bp DNA ladder
Lane 2: control positive
Lane 3: control negative
Lane 5:positive isolates to *P. multocida*

DISCUSSION

Mycoplasmas are considered to be one of the pathogens causing BRD. They are able to cause mild respiratory disease by themselves, but more often they are isolated from pneumonic lungs together with other pathogens *(23- 25)*.

In this study , as shown in Table (2) *Mycoplasma bovis* was recovered from nasal swabs and pneumonic lungs with prevalence (8% and 6%) respectively , these results more or less near with the result obtained by ***(19 ,26)***. Molecular diagnosis of *Mycoplasma bovis* are rapid, simple, accurate as reported by *(27)* that the use of PCR makes the identification of *M. bovis* much shorter comparing to the conventional culture methods. As shown in Fig (1).

Pasteurella multocida is another common Pathogen reported to be involved in BRD, especially in dairy herds ***(28,29)*** As shown in Table (2): *Pasteurella multocida and Mannheimia haemolytica* were recovered in a percentage (18% and 12%) and (14% , 10%) in case of nasal swabs and lung tissues , respectively, these results agreed with these of ***(30- 33),*** they reported that dairy calf pneumonia is multifactorial in nature , stress , housing , ventilation , colostral immunity , and a number of viral agents have been proposed to play an important role in initiating this disease complex . *Mannheimia haemolytica and pasteurella multocida* are frequently isolated from the purulent bronchopneumonia lung associated with this disease. While these organisms are the primary bacterial agents of BRD, they are also part of the normal upper respiratory flora of calves. Many microorganisms have the ability to vary their size, shape and surface antigens further complicating identification occurred by serological and biochemical assays ***(34)***. 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 ***(35, 36)***. 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 *(37,38)*.

In the present study the samples of nasal swabs and samples of lung tissues of diseased cattle suffered from respiratory symptoms were mycologically and bacteriologically investigated. The results of fungal contamination revealed that the yeast of *Candida sp.* was the most predominant in all cases (56% and 40%), respectively. The recovery of yeast fungal contamination in association of respiratory affection of cattle were previously reported by ***(39- 42)*** whereas, the other genera that isolated from some cases were recovered in various order of frequency. The fungus of *Penicilium sp.* was recovered from 32% of nasal swabs and 24% of lung tissues samples. *While, A.niger, A.flavus, A. candidus and A.fumigatus* were also isolated from 72, 16, 16, 8% of nasal swabs of diseased cattle where Aspergillosis is caused by several *Aspergillus sp.*, especially *A. fumigatus* , *A. niger*, *A. flavus* are being recognized more commonly as molecular techniques for identification are being increasingly used. Aspergillus infection is found worldwide and in almost all domestic animals as well as in many wild species. It is primarily a respiratory infection that may become generalized; similarly, ***(43),*** investigated forty diseased and apparently healthy cases of cattle and revealed that the nasal swabs of diseased animals had Aspergillus *spp* as the predominant cause from the above samples of infected animals, where in cattle isolated from (96%) Other genera and species of moulds and yeast were obtained at comparatively lower significant rates.

In affected cattle, infections with Aspergillus may be asymptomatic. In respiratory aspergillosis, respiratory symptoms such as coughing, dyspnea and hemoptysis may be apparent. In some cattle, this can be rapidly fatal as dissemination of spores occurs through the pulmonary circulation.

CONCLUSION

From the achieved results of the present study, it could be concluded that *Mycoplasma bovis*, *pasteurella multocida*, *M . haemolytica* and Fungi represent the main causes of bovine respiratory disease of cattle in Egypt. Fungi typically enter the lung of animals with inhalation of their spores, though they can reach the lung through the blood stream if other parts of the body are infected. Thus leading finally to bacterial and fungal pneumonia. In addition molecular diagnosis consider reliable, sensitive and rapid methods depending upon the extraction of adequate amounts of pure DNA using appropriate methods.

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الملخص العربى

دراسة عن بعض المسببات الفطرية والبكتريه والميكوبلازميه لمرض الالتهاب الرئوي فى الأبقار

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مرض الالتهاب الرئوي في الأبقار يسببه مسببات ميكوبلازميه ، بكتيرية وفطرية و هو يسبب مشكلة صحية كبيرة للماشية في جميع أنحاء العالم. وقد أجريت هذه الدراسة لفحص ١٠٠ عينه مسحات أنفيه أخذت من أبقار تتراوح أعمارها من ١- ٥ سنوات من مزارع خاصة بمحافظة المنوفية تعانى هذه الحيوانات من خمول ونقص في كمية الطعام المتناول ، مشاكل تنفسيه ، ضيق فى التنفس مع ظهور وعدم ظهور رشح من الأنف والتهاب رئوي. و كذلك تم أخذ ٥٠عينه من رئات مصابه تم جمعها من مجازر مختلفة في محافظة المنوفية والتي تم الحصول عليها من أجزاء يظهر عليها علامات الالتهاب الرئوي.

تم عزل الميكو بلازما بوفس ، البستيرللا ملتوسيدا و مانهيما هيمولتيكا من مسحات الأنف بنسب (٨، ١٨ و١٢ %) ، كما عزلت من الرئات المصابة بنسب (٦ ، ١٤ ، ١٠%) لكل منهما على التوالي

كانت أهم المعزولات الفطرية الاسبرجلس فلافس ، الاسبرجلس نيجر ، الاسبرجيلس فيوميجيتس ، الاسبرجيلس كانديدس ، فطر البنيسيليوم و خمائر الكانديدا بنسب (١٦ ، ٧٢ ،١٦، ٨ ، ٣٢ و 56%) و(٨ ،٨ ،٠٠، ٨ ، ٢٤ و٤٠%) لكل من المساحات الانفيه والرئات على التوالي.

يعتبر اختبار تفاعل أنزيم البلمرة المتسلسل (PCR) هو محدد للغاية، بسيط وحساس و سريع التشخيص.وقد تم أجراءه لكل من الميكوبلازما بوفس ،البسيريلا مالتوسيدا، اسبرجيلاس فلافس ، اسبيرجلاس نيجر ، اسبيلاجلاس فيوميجيتس وخمائر الكانديدا. كما تم عمل اختبارات الحساسيه لمعزولات البستيريلا وكانت المعزولات حساسه لكل من الدراكسين والجنتاميسن ،اما معزولات مانهيما هيمولتيكا كانت حساسه للدراكسين ، بينما كانت معزولات الميكوبلازما حساسة لكل من الدراكسين والسيبروفلوكساسين.