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Molecular study on *Mannheimia haemolytica* and *Aspergillus fumigatus* causing respiratory affection in buffalo calves

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Abstract

Respiratory diseases affecting buffalo calves are considered the main threat to their breeding and production, causing severe losses among these animals. *Mannheimia haemolytica* and *Aspergillus fumigatus* are the main bacterial and fungal species causing respiratory infections in buffaloes, respectively. A total of 50 samples, 25 nasal swabs and 25 lung tissues, were collected from diseased and slaughtered buffalo calves, respectively. The samples were subjected to bacteriological examination for detecting the prevalence of *M. heamolytica* and mycological examination for detecting the prevalence of *A. fumigatus* and other *Aspergillus* species. Results obtained showed that the prevalence of *M. heamolytica* was 18% (9 samples), 12% of which were isolated from infected lung tissues (6 samples) and 6% were isolated from infected nasal swabs (3 samples). While the prevalence rate of isolation of fungi was 34% (17 samples), including *A. fumigatus* (9 samples), *A. niger* (5 samples) and *A. flavus* (3 samples). Application of PCR procedure for confirmation of two *M. heamolytica* isolates which were identified biochemically as typical *M. heamolytica* revealed that two isolates were positive for *ssa* gene and gave amplicons at 325 bp. Virulence gene (*aspHS*) of three *A. fumigatus* isolates were detected by PCR, only one of which gave amplicon at 180 bp. Antibiogram susceptibility test of the isolated *M. heamolytica* species showed that the isolates were highly sensitive to ceftiofur and florfenicol followed by ampicillin and penicillin while *A. fumigatus* isolates were sensitive to terbinafin and itraconazole.

Keywords: *Mannheimia haemolytica, Aspergillus fumigatus, isolation, identification, PCR, buffaloes*

Introduction

Bovine respiratory disease (BRD) is a general term for respiratory disease in cattle caused by a range of factors, singly or in combination. A major cause of economic losses, BRD affects the lower respiratory tract / lungs (pneumonia) or upper respiratory tract (rhinitis, tracheitis, bronchitis). Bovine respiratory disease in cattle is an interaction between the infectious agents (bacterial, viral and fungal), the environment, and the immunity of the individual animal **(Allan *et al.*, 1991)**.

Mannheimia haemolytica is an important etiological agent in bovine respiratory disease **(Noyes *et al.*, 2015)**. It is the bacterium most commonly associated with the gross pathology of the acute fulminating bronchopneumonia, typically seen within the first few weeks that cattle are on feed **(Confer, 2009)**. *Mannheimia heamolytica* is a major bacterial component of bovine respiratory disease, unfortunately very little is known about *Mannheimia haemolytica* transmission dynamic among cattle (**Sarah *et al.*, 2015)**.

Mannheimia haemolytica is the principal bacterium isolated from respiratory disease in feed

lot cattle and is a significant component of enzootic pneumonia in all neonatal calves **(Rice *et al.*, 2007)**. It has been considered one of the predominant bacterial pathogens associated with bovine respiratory disease **(Rice *et al.*, 2007)**. Extensive work focusing on the characterization of virulence factors in *Mannheimia haemolytica* **(Highlander , 2001 and Zecchinon *et al.*, 2005)** and the recent sequencing of the *Mannheimia haemolytica* genome **(Gioia *et al.*, 2006 and Lawrence *et al.*, 2010)** has helped elucidate much of the genetic basis for its pathogenesis. However, most of these studies have focused on serotype 1 isolates or are limited to only a few strains.

The disease is determined by specific serotypes found in bovine. The molecular epidemiology of strains involved in disease is important in the control of outbreaks as well as in the preparation of vaccines **(Villard *et al*., 2008).**

Aspergilli are predominantly saprophytes, growing on dead or decaying matter in the environment. The infectious life cycle of Aspergillus begins with the production of conidia (asexual spores) that are easily dispersed into the air, ensuring ubiquity in both indoor and outdoor environments **(Falvey and Streifel, 2007)**. The primary route of cow's infection is via inhalation or consumption of *Aspergillus fumigatus* conidia**.** These infections become invasive with the activation of the conidia to a hyphal form which initiates active destruction of tissue in the lungs and gastrointestinal tract and movement of the fungus into surrounding tissue **(Stanzi, *et. al.,* 2005)**, followed by conidial deposition in the bronchioles or alveolar spaces. In healthy individuals, conidia that are not removed by mucociliary clearance encounter epithelial cells or alveolar macrophages, the primary resident phagocytes of the lung. Alveolar macrophages are primarily responsible for the phagocytosis and killing of Aspergillus conidia as well as the initiation of a pro-inflammatory response that recruits neutrophils (one type of polymorphonuclear leukocytes) to the site of infection. Conidia that evade macrophage killing and germinate become the target of infiltrating neutrophils that are able to destroy hyphae. The risk of developing invasive Aspergillosis results primarily from a dysfunction in these host defences in combination with fungal attributes that permit A. fumigatus survival and growth in this pulmonary environment **(Morris *et al*., 2000)**

The polymerase chain reaction (PCR) was used to detect *Pasteurella multocida* and *Mannheimia haemolytica* directly from clinical samples (**Deressa *et al.*, 2010)**. It facilitates in vitro amplification of the target sequence. The advantages of PCR are vast. Organisms do not need to be cultured, at least not for long period prior to their detection. The target DNA can be detected easily even in a complex mixture, with no radioactive probes required. In addition, PCR is a rapid, sensitive and highly versatile procedure **(Logotheti *et al*., 2009)**. The Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) are highly variable sequences of great importance in distinguishing fungal species by PCR analysis. Previously published PCR primers available for amplifying these sequences from environmental samples provide varying degrees of success at discriminating against plant DNA while maintaining a broad range of compatibility. Typically, it has been necessary to use multiple primer sets to accommodate the range of fungi under study, potentially creating artificial distinctions for fungal sequences that amplify with more than one primer set **(Martin and Rygiewicz, 2005).**

The aim of the current study was to investigate the role of *Mannheim haemolytica* and *Aspergillus fumigatus* as primary causes of infections in diseased buffalo calves with respiratory manifestations and slaughtered ones with pneumonic lesions. Molecular studies of the isolates to detect the virulence genes, in addition to antibiogram sensitivity tests of the isolated causative agents.

Materials and Methods

Samples:

A total number of 50 samples were collected from buffalo calves, 25 of which were nasal swabs collected from diseased calves showing respiratory manifestations. The other 25 samples were lung tissues collected from diseased calves showing pneumonic lesions at slaughtering house. Samples were collected in sterile bags for bacteriological and mycological examination.

Bacteriological examination (Quinn *et al.*, 2011)

Swab samples were cultured directly onto blood agar and MacConkey agar plates while the lung tissue samples were streaked onto blood and MacConkey agar plates. All plates were incubated aerobically at 37ºC for 24-48 hours. The growing surface colonies were identified by cultural, morphological and biochemical characteristics. The isolates were also identified using Vitek 2 compact system (Biomerx) according to the manufacturer manual **(Chatzigeorgiou *et al.*, 2011)**.

Mycological examination

The nasal swabs were directly cultured by streaking on the surface of plate of Sabouraud Dextrose Agar media (SDA) containing chloramphenicol 0.05 mg/ml, inoculated plates were incubated at 25ºC in case of moulds for 5 -7 days. While for the lung samples, they were immersed in 70% ethyl alcohol for 3 minutes to remove the external contamination and then the samples were opened and the contents were inoculated onto SDA media. All isolated filamentous fungi were cultivated on appropriate media for their identification according to **(Klich and Pitt, 1988; Klich, 2002; Samson and Frisvad, 2004)**.

Proteolytic activity for Aspergillus fumigatus

Casein hydrolysis was done on modified Czapek agar (MCA) medium according to **(El-Fadaly *et al.*, 2015)**. One ml of sterilized skimmed milk was added to modify Czapek agar (MCA) medium. All plates were spot inoculated by *A. fumigatus* isolated strains. After incubation at 25˚C, the plates of casein were flooded with copper sulfate (10%).The results were recorded by measuring the diameter of growth and clear zone.

PCR procedure for *M. heamolytica*

DNA extraction: DNA extraction from two randomly positive isolates for *M. hemolytica* by Vitek 2 was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56ºC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primers: Primers used were supplied from Metabion (Germany) and listed in **Table 1**.

PCR amplification: Primers were utilized in a 25 µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products: The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5 V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. Gelpilot 100 bp ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1). Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
ssa	TTCACATCTTCATCCTC TTTTCATCCTCTTCGTC	325	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 40 sec.	72˚C 10 min.	**Hawari *et al*., (2008)**

DNA extraction and PCR amplification of *Aspergillus fumigatus* (Logotheti *et al*., 2009 and Diaz-De-Cerio *et al*., 2013)

Genomic DNA extracted from *A. fumigatus* which gave high proteolysis (1.5 mm) was obtained using the genomic DNA Extraction Kit (Axygen Plant Genomic DNA Extraction Kit, catalog number 14-223-418, USA) following the manufacturer's instructions. DNA concentration was determined spectrophotometrically at 260/230 nm. The PCR primers used in this study of *A. fumigatus* virulence gene were provided by Analysis Company and listed in **Table 2**. The PCR reaction was performed in a Gradient Thermal cycler (S-1000 Thermal cycler Bio-RAD, USA). The reaction mixture (total volume of 50 µl) was 25 µl Dream green PCR Mix (DreamTaq Green PCR Master Mix (2X) Fermentas Company, cat., No.K1080, USA.), 5 µl target DNA, 2 µl of each primer (containing 10 pmole/ µl) and the mixture was completed by sterile distilled water to 50 µl.

The PCR amplification protocol used for *A. fumigatus* virulence gene: Initial denaturation at 95ºC for 10 min followed by 32 cycles of 95 ˚C for 1 min, 60ºC for 1 min, and 72ºC for 1 min, and a final step at 72 ˚C for 10 min. Amplification products were electrophoresed in agarose gels (2% w/v) (Agarose, Sigma, USA), stained with ethidium bromide using Gene Ruler 100bp DNA Ladder (Fermentas Company, Cat. No. SM0243, USA).

Table (2). Primer names, sequences and amplicon size used

Species	Primer pairs	Sequence (5′→3′)	Amplicon size (bp)
Virulence gene of *A. fumigatus*	F-aspHS R-aspHS	TGGTACAAGGACGGTGACAA- GTCCCAGTGGACTCTTCCAA	180

PCR reaction mixes, amplification and PCR product analysis were performed in different rooms. Specific plastic disposable material, filtered tips and automatic pipettes were used for each step to avoid contaminations. In addition, PCR reaction mixes were performed in a laminar flow cabinet and all surfaces were cleaned with Termi-DNA-tor.

Antibiotic sensitivity test for *M. haemolytica* isolates

It was done by the disc diffusion method and the results were interpreted according to NCCLS **(NCCLS 2002)**.

Antifungal susceptibility testing

The isolated *A. fumigatus* was subcultured on SDA and incubated at 37ºC for 5 days. In vitro, the sensitivity of the isolate to antimicrobials was determined according to standards of National Committee for Clinical Laboratory (**NCCLS, 2002)**. A loopful from pure culture of *A. fumigatus* was mixed well with 9 ml of sodium chloride solution, spread over the surface of SDA plate, and the excess fluid was sucked. Four antifungal discs **(griseofulvin,**

terbinafine, fluconazole and itraconazole) were spread on the surface of the inoculated plate. Plates were incubated at 37ºC for 5 days. The diameter of inhibition zone of each disc was measured in mm and evaluated.

Results & Discussion

Respiratory problems among calves are considered the most serious infections affecting them which may be brought on by lack of proper management, over rowdiness and competition for food. Additionally, the presence of calves in close premises and even the yards of calves close and near to each other facilitate the transmission of diseases among the animals. Also, animal keepers have great responsibility in spreading the infection during their daily routine work **(Radostits *et al*., 2007)**. Globally, the susceptibility of calves to pneumonia is higher during the first 1-3 months of age than at any other time. Instances of pneumonia vary based on the conditions where the calves are held. Housing systems, feeding, climate and management are some examples of the factors that influence calves' susceptibility **(Meglia *et al*., 2001).**

Mannheimia haemolytica is an opportunistic Gram-negative bacterium that belongs to the Pasteurellaceae family. It is considered as a respiratory pathogen affecting cattle and related ruminants worldwide. *M. haemolytica* is commonly associated with bovine respiratory disease complex (BRDC), a polymicrobial multifactorial disease **(Harhay *et al.*, 2013)**.

Most of respiratory infections with bacteria are not capable of inducing significant disease without the presence of other predisposing environmental factors, physiologic stressors, or concurrent infections **(Griffin *et al.*, 2010)**.

Fungal infections are often observed in animals whose lungs are simultaneously colonized with bacteria. The consequences of co-infections by bacteria and fungi are not well known, co-infections indicate that this category of infections correlates with decreased lung function when compared to infections by either bacteria or fungi alone **(Leclair *et al.*, 2010)**.

As shown in **Table 3**, *Mannheimia haemolytica* was isolated from 9 infected calves (18%), diseased and slaughtered, with the isolation of the organism from 3 nasal swabs and 6 pneumonic lung tissues. Identification of the isolates by traditional methods gave the same results of using Vitek 2 compact system for identification. While for Aspergillus species, the prevalence of mycotic pneumonia in calves was 24% in nasal swabs and 44% in lung tissues. Out of the infected samples, 2 nasal swabs contained mixed infections from *M. heamolytica* and Aspergillus species and only one lung tissue sample contained mixed infections.

Table (3). Prevalence of *M. heamolytica* and Aspergillus species in the examined buffalo calves samples

Type of samples	No. of examined samples	Samples +ve for *M. haemolytica*		Samples +ve for *Aspergillus sp.*		Samples containing mixed infections
		No.	%	No.	%	No.
Nasal swabs	25	3	12	6	24	2
Lung tissues	25	6	24	11	44	1
Total	50	9	18	17	34	3

% calculated according to the No. of samples examined

A study, conducted in Egypt, found that the occurrence of *M. haemolytica* in buffaloes was 20% (**Kaoud *et al.*, 2010)**. Furthermore, a study in 2007 recorded that the prevalence of *M. haemolytica* in the diseased Egyptian cattle was 8% (**Ilhan and Keles 2007)**. This may suggest the influence of bacterial factors, source of isolation, different isolation techniques, misidentification, virulence, localities and season effects in the epidemiology of *M. haemolytica* infection in ruminants. On the other hand, the results of Aspergillus sp. agree with several studies, one of which recorded that pulmonary aspergillosis was found in 10 cases that represented 52.6% **(Chihaya *et al.*, 1991)**. Another study recorded that the total mycological isolations from lung tissues were 116 cases, representing 58% **(Abeer *et al.*, 2011)**. Aspergillus sp. is prevalent worldwide and their spores are frequently present in the biosphere, so they are part of routinely inhaled particles.

Aspergillus fumigatus, the main causative agent of invasive aspergillosis, is a saprophyte that grows and sporulates in a humid environment on decaying organic materials. Although this species is not the most prevalent fungus in the world, it is one of the most ubiquitous of those with airborne conidia. It speculates abundantly, with every conidial head producing thousands of conidia. The conidia released into the atmosphere have a diameter small enough (2 to 3 μm) to reach the lung alveoli. *A. fumigatus* does not have an elaborate mechanism for releasing its conidia into the air; dissemination simply relies on disturbances of the environment and strong air currents. Once the conidia are in the air, their small size makes them buoyant, tending to keep them airborne both indoors and outdoors. Environmental surveys indicate that animals will inhale at least several hundred *A.fumigatus* conidia per day **(Hospenthal *et al.*, 1998)**. Like many other ascomycetes fungi, *A. fumigatus* grows well in a medium containing protein as the sole nitrogen and carbon source and secretes proteolytic enzymes which could be an important contributor to this virulence capability **(Sriranganadane *et al.*, 2011)**.

From the results in **Table 4**, it is evident that the isolated mould species were 24% & 12% for *A. fumigatus*, 12% & 8% for *A. niger* and 8% & 4% for *A. flavus*. Nearly similar results were found by other studies **(Abeer *et al.*, 2011; Zlotowski *et al.*, 2011)**. One study recorded that among lung samples from buffalo calves, 35% contained *A. fumigatus* and 20% *A. niger* (**Shawky *et al.*, 2014)**.

Table (4). Incidence of Aspergillus species isolated from examined samples.

Isolates	Lung samples (n=25)		Swabs samples (n=25)	
	No.	%	No.	%
A. fumigatus	6	24	3	12
A. niger	3	12	2	8
A. flavus	2	8	1	4

Results of proteolysis were presented in **Table 5** and **Figure 1**. All tested *A. fumigatus* strains showed positive results. The fungal growth diameters were corresponding to clear zone diameter and it was varied between 1.5 to 1.1 mm in the cultures of *A. fumigatus* strains. Also, it is noted that the strains isolated from the lung samples showed their high ability for proteolysis.

Table (5). Efficiency of A. fumigatus strains for proteolysis.

***A. fumigatus* strains isolates**		**Clear zone diameter (mm)**
Lung	Nasal swab	
Strain 1	**---**	**1.5**
Strain 2	---	**1.2**
Strain3	---	**1.5**
Strain4	---	**1.1**
Strain5	---	**1.2**
Strain6	---	**1.5**
---	**Strain7**	**1.2**
---	**Strain8**	**1.2**
---	**Strain9**	**1.1**

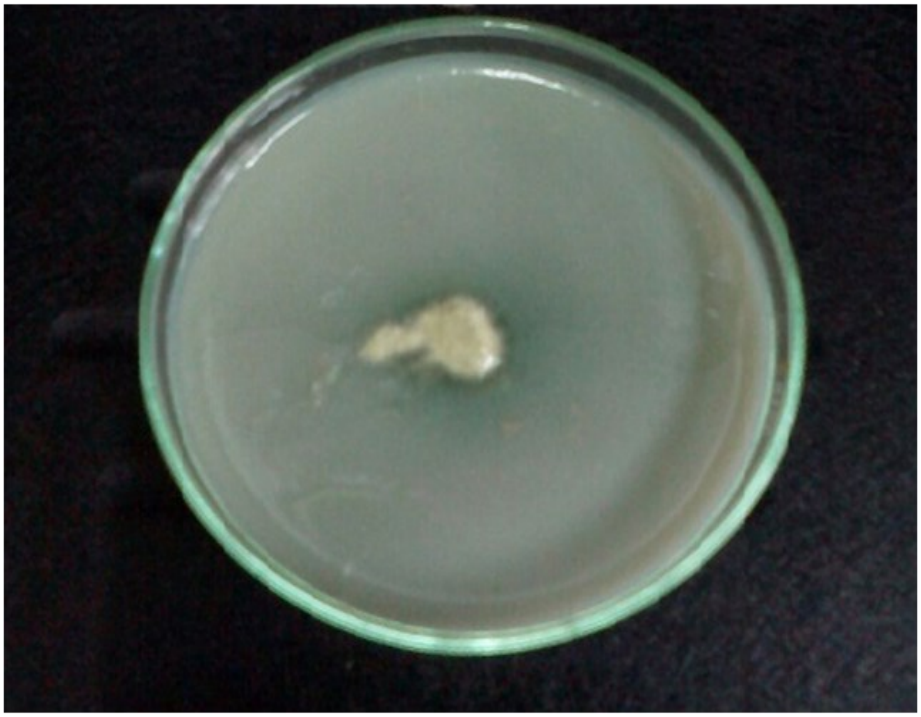

Figure (1). Proteolytic activity of *A. fumigatus* on modified Czapek agar (MCA) medium

Using a molecular genetic technique approach, the genes that code for the various virulence factors of *M. haemolytica* A1 have been cloned for detailed characterizations. These included analysis of the encoded protein, their biological activities, secretion of the molecules from the bacterium as well as their use in a vaccine component **(Lo, 2001).** In our study, ssa gene of *M. haemolytica* was detected in the two tested isolates, as shown in **Figure 2 ,** and gave amplicons at 325 bp.

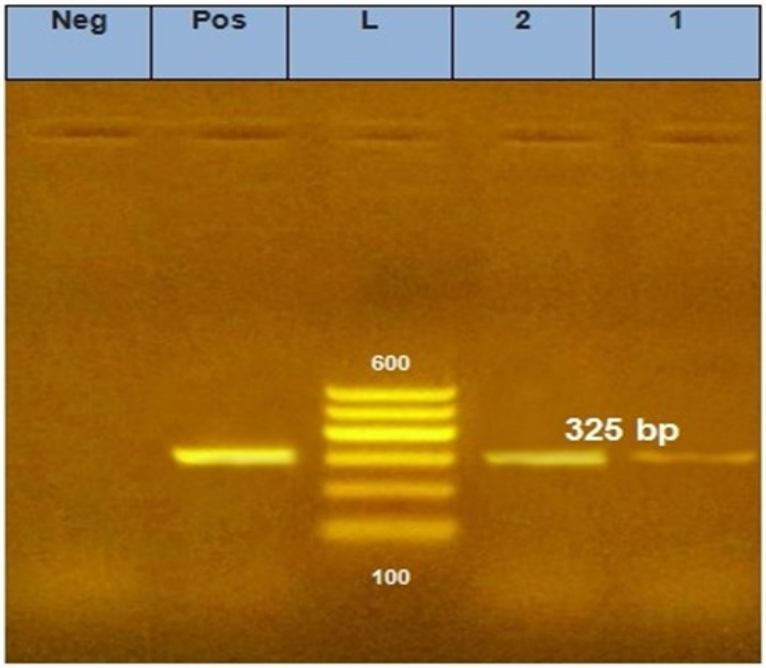

Figure (2). Agarose gel electrophoresis for *Mannheimia haemolytica*
Neg: *M. haemolytica* negative control; POS: *M. haemolytica* positive control;
L: 100 bp DNA ladder; 2-1: positive samples of *M. haemolytica* at 325 bp

Diagnosis of aspergillosis can be complex requiring interpretation of clinical, radiological and microbiological–serological findings. On the other hand, timely administration of the proper antifungal therapy is imperative for the treatment of these infections, which are usually severe. Certain criteria for the diagnosis of invasive aspergillosis have been established and various diagnostic methods aiming at the early detection of aspergillus have been evaluated. Nevertheless, the conventional microbiological methods including culture, biochemical identification, immunoassay and microscopic examination are time-consuming and lack sensitivity (**Logotheti, *et al.*, 2009)**.

The polymerase chain reaction (PCR) has already been used to detect DNA from a number of pathogens when diagnostic methods are limited and it is used to amplify specific sequences of DNA from *A. fumigatus* followed by agarose gel electrophoresis. *A. fumigatus* seems to possess an exclusive combination of different virulence related factors, making it the leading mould pathogen in the world. Analysis of these virulence factors could give the scientific community new clues to the understanding of its pathogenicity and improve the selection of targets for clinical diagnosis. In our study research, selected virulence related *aspHS* gene showed that it could be successfully used for specific identification of *A. fumigatus* by conventional PCR. This gene encodes for a haemolysin, with activity against rabbit and sheep erythrocytes and cytotoxic effects on macrophages and endothelial cells in vitro. The *aspHS* gene is more highly expressed in vivo than in vitro and it has also recently been reported as a major in vitro-secreted protein **(Gravelat, *et al*., 2008 and Wartenberg, *et al*., 2011)**.

In **Figure 3**, electrophoresis on agarose gel (2%) of 7 µl of products obtained from *A. fumigatus* to detect virulence gene (*aspHS* gene) by PCR technique was shown. Lane 1 in the figure detected the 100 bp DNA ladder, while Lane 2 showed the positive control of *A. fumigatus*. Lane 3 was the negative control. Lane 4 showed the sample positive for *A. fumigatus* at 180 bp while the last 2 lanes (5 and 6) were negative for *A. fumigatus*.

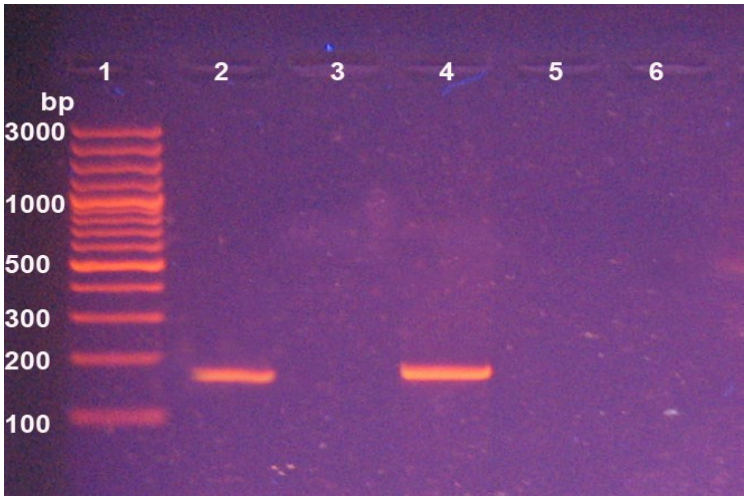

Figure (3). Agarose gel electrophoresis for *Aspergillus fumigatus*
Lane 1: 100 bp DNA ladder; Lane 2: *A. fumigatus* control positive; Lane 3: Control Negative; Lane 4: positive sample of *A. fumigatus*at 180 bp; Lane 5-6: Negative samples of *A. fumigatus*

Application of sensitivity test for the isolated *M. haemolytica* is illustrated in **Table 6.** All isolates were found to be sensitive to ceftiofur and florfenicol (100%) while the isolates were resistant to tetracycline, amoxicillin and gentamycin. The other used antibiotics showed variation in their sensitivity. One study reported that *M. heamolytica* isolates were highly sensitive to ceftiofur and florfenicol and moderately sensitive to gentamycin and streptomycin while the isolates were resistant to tetracycline and amoxicillin, which was in accordance with our results **(Mevius and Hartman 2000)**. Also another study reported that there was resistance of *M. haemolytica* isolates to oxytetracycline, which is a derivative of tetracycline **(Alexander *et al.*, 2013)**.

Table (6). Antibiotic sensitivity test of the isolated M. heamolytica strains (n=9)

Antibiotic	Code / Potency (µg)	Sensitive n	Resistant n
Ampicillin	AMP/10	7	2
Amoxicillin	AMX/10	3	6
Cloxacillin	OB/5	6	3
Penicillin	P/10	7	2
Erythromycin	E/15	6	3
Tetracycline	TE/30	2	7
Ceftiofur	EFT/30	9	0
Florfenicol	FFC/30	9	0
Gentamycin	CN/10	3	6
Streptomycin	S/10	5	4
Enrofloxacin	ENR/5	6	3

The results presented in **Figure 4** showed that terbinafine and itraconazole were of similar effectiveness and more effective than fluconazole in inhibiting *A. fumigatus,* while *A. fumigatus* showed resistance to griseofulvin.

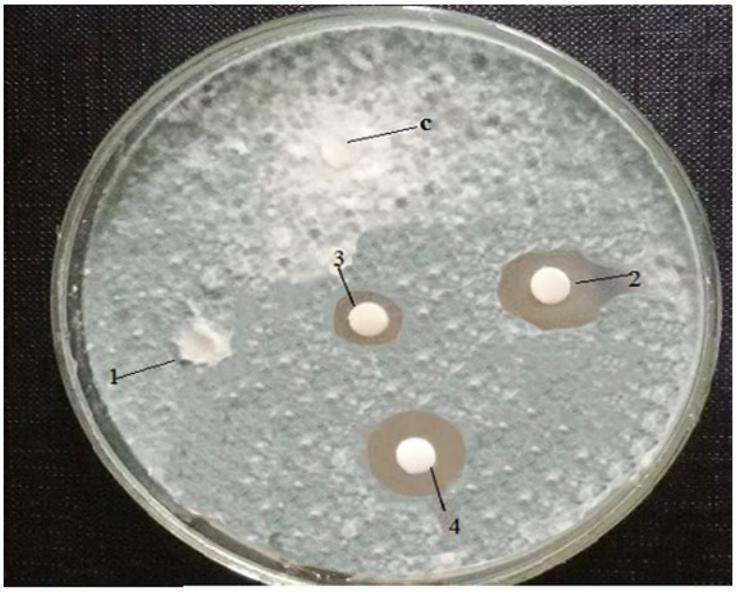

Figure (4). Sensitivity of *A. fumigatus* to antifungal drugs
C= control, 1= Griseofulvin (-), 2=Itraconazole (+++), 3= Fluconazole (++), 4=Terbinafine (+++)

Conclusion & Recommendation

According to the present study, it could be concluded that *Mannheimia haemolytica* and *Aspergillus fumigatus* are main causes of respiratory infections in bovine, particularly calves of low immunity. Both cause the disease in a mixed infection and as single infection in the study. Ceftiofur and florfenicol are the drugs of choice for treatment of *M. haemolytica* while for *A. fumagitaus*, itraconazole and terbinafine are the antifungal drugs of choice.

Application of strict hygienic measures in animal farms should be done by periodical application of disinfectants to protect animals from respiratory diseases causing agents, as bacteria, viruses and fungi. Sudden exposure of animals, especially in closed system of breeding, should be avoided as it is a predisposing factor for respiratory infections. Periodical clinical examination of animals to detect early infections should be performed. Misuse of antibiotics should be avoided and only used according to the lab results. Annual vaccination of animals against *M. haemolytica* is also recommended.

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