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Histopathological, Bacteriological and Molecular study of Small Ruminants Enzootic Respiratory Complex at Al Muthanna governorate

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Abstract

Small ruminants play an essential part in the nutrition and income of people around the world as well as in Iraq. The production of the small ruminant in Iraq is affected by diseases, inadequate nutrition and poor genetic resources of the local stock. High morbidity and mortality rates in small ruminants occur due to respiratory tract diseases in Iraq. Consequently, this study intends to investigate the bacteriological and histopathological features of small ruminant's enzootic respiratory complex in Al Muthanna governorate, moreover, to characterize the isolated *Mannheimia haemolytica* using PCR technique. The study was extended from October 2017 to March 2018, as a cross-sectional survey on sheep and goats. One hundred four nasal swabs collected from the nasopharyngeal area from sheep and goats. The nasal swab was analyzed using standard methods. Tissue samples were also collected from animals for histopathological investigation. Molecular Identification was done for isolated *Mannheimia haemolytica*. A total number of diseased animals was 104 out of 270 (38.51 %), in addition to the five dead animals. The percentages of the respiratory diseases were 57.94% and 25.76% in sheep and goat respectively. *Mannheimia haemolytica*, *Escherichia coli*, *Pasteurella multocida*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, and *Streptococcus pyogenes* were isolated. Suppurative and exudative pneumonia, congestion, and various stages of pneumonia were seen grossly. Histologically, suppurative, necrotic and fibrinous bronchopneumonia, and bronchointerstitial and pyogranulomatous pneumonia were seen. All *M. haemolytica* isolates were positive in PCR for 16 s rDNA and 12 s rRNA genes that showed a specific 304 bp and 270 bands respectively on the agarose gel. In conclusion, this study approved the incidence of small ruminant's enzootic respiratory complex in Al-Muthanna governorate. Moreover, *M. haemolytica* showed positive results with PCR. The authors recommend considering PCR as a valuable tool for the rapid detection of *M. haemolytica*. Another epidemiological studies needed to be done regarding the role of *M. haemolytica* and other causative agents in clinical cases in small ruminants enzootic respiratory complex, thereby providing the basis for effective preventive strategies.

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Keywords: Al Muthanna governorate, *Klebsiella pneumoniae*, *Mannheimia haemolytica*, small ruminant's enzootic respiratory complex, *Staphylococcus aureus*.

Introduction

Small ruminants are valuable possessions for the Middle East, Southeast Asian and African countries with the possibility of providing wool, milk, and meat. Small ruminants are highly prone to the diseases of the respiratory system, which lead to 50% mortality between them (Lacasta *et al.,* 2008; Pugh, 2002). The diseases of the respiratory system of small ruminants lead to 5.6 % of the total diseases of the small ruminants. The respiratory disorders are divided into two groups: 1. the diseases of the upper respiratory tract that including sinusitis and caused by the larvae of parasites, nasal foreign bodies, gaseous irritation, and enzootic nasal tumors; 2. the diseases of the lower respiratory tract that contain mainly pneumonia, moreover, bacterial, viral or and fungal are the most infectious origin (Woldemeskel *et al.,* 2002; Pugh, 2002). Meanwhile, the environmental pollutants, toxicants, and mechanical induction of respiratory distress may play a role in these abnormal conditions. Depending on the physiological, environmental, and etiological factors, respiratory conditions might be acute, chronic, and progressive (Kumar *et al.,* 2000; Soni and Sharma, 1990). To overcome such essential disease conditions, data on their identification, prevention, cure, and control can enhance and encourage the economic status and sustainability of holders of small ruminants. Thus, an early, rapid, and specific diagnosis of such diseases give great importance to decrease the losses. The advanced enzyme-linked immunosorbent assays (ELISAs) for the detection of antigens as well as antibodies directly from the samples are primarily available for all the diseases with specificity and sensitivity likely, molecular diagnostic assays along with microsatellites comprehensively help in diagnosis as well as treatment and epidemiological studies (Brogden *et al.,* 1998; Hindson and Winter, 2002; Garedew *et al.,* 2010; Scott, 2011). It is necessary to apply correct control and prevention protocols and devising suitable control strategies to overcome such important respiratory diseases, therefore alleviating the economic losses (Scott, 2011; Bell, 2008). Some pathogenic microorganisms have been accused in the development of respiratory diseases, but the importance of environmental factors in the initiation and progress of the disease can never be overlooked. All these environmental factors irritate the respiratory system producing stress in the microenvironment leading a decrease in the immune status of the animals and thereby helping bacterial, viral, and parasitic infections in breaking down the tissue defense barriers. Environmental pollutants lead to acute or chronic reactions as they deposit on the alveolar surface, which are characterized by inflammation or fibrosis and the express of transitory or persistent tissue manifestation. The disease development can be portrayed as three sets of two-way communications among pathogen, environment, and host but the interactions are highly variable. Additionally, the environmental scenario is never static; new compounds are introduced daily making a precise evaluation of the disease burden almost impossible (Daniel *et al.,* 2006; Pavia *et al.,* 2011). It assumes a uniquely influential position in livestock production. Small ruminants can affect remarkable adaptability to diverse environmental conditions and are amenable ease of

management. They are thus, a reliable source of income and cash security. Furthermore, they provide meat, skin, wool, and manure that maintain soil fertility.
In Iraq, small ruminants play a significant role in the Iraqi national economy. It is estimated 9,900,000 Head of sheep and goat in 2014 (https://en.actualitix.com/country/irq/iraq-livestock-of-sheep-and-goats.php; Al-Salihi, 2012). They supply a high percentage of all domestic meat consumption. Therefore, an increase in small ruminant production is needed to maintain self-sufficiency. Small ruminant production in the country, however, is still constrained by various factors. The major constraints facing sheep and goats production include disease, inadequate nutrition, poor genetic potentials of the local stock, marketing, social factors, structural constraints and the shortage of high level of trained manpower (Kumar *et al.*, 2000). Of these, multifactorial infectious diseases of small ruminants cause substantial loss through morbidity and mortality (Soni and Sharma, 1990). The bacterial infection of the respiratory tract may be primary, occurring in healthy individuals or secondary to a large number of conditions, that weaken the animal resistance. Secondary bacterial infection occurs mainly when the local resistance of the respiratory mucosa is lowered, and bacterial growth in the nose and throat extends downwards, usually giving a mixed infection (Kumar *et al.*, 2013).
Review of literature concerning small ruminants enzootic respiratory complex at Al-Muthanna governorate revealed scarce publications. Consequently, this study designed to investigate the bacteriological and histopathological features of small ruminants enzootic respiratory complex at Al-Muthanna governorate. moreover, to characterize the isolated *Mannheimia haemolytica* using PCR technique.

Materials and methods

Samples collection and bacterial culture

This study was conducted in Samawah city / Al Muthanna Government/Iraq during a period started from October 2017 to March 2018. A study was done as a cross-sectional survey on the slaughtered sheep and goats in Al Muthanna abattoir (Figure.1), and small ruminants admitted to veterinary hospital with respiratory signs. In abattoir, all animals were examined for any signs of respiratory diseases before slaughtering. One hundred four swabs from the nasopharyngeal area were collected from sheep and goats that showed nasal discharge. (Figure.2).

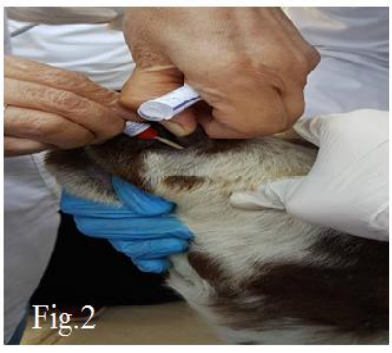
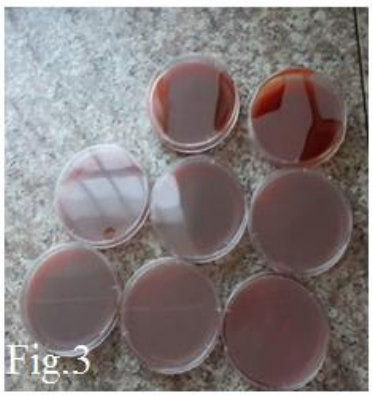


Figure. 1: Shows the examination of the animal before slaughtering
Figure.2: Shows nasal swab collection from the infected sheep.

After well cleaning and disinfection of the external nares, the nasal samples were collected by introducing sterile cotton swab into the nasal passage. All nasal swabs were carefully put into a labeled bottle containing 2 mL brain heart infusion broth. Then, the swabs were transported in a cool box to the laboratory for bacterial culture. Moreover, lung and tracheal samples were also collected from 5 dead animals in 2 herds of sheep that suffered from high morbidity and mortality rates due to respiratory infection. After the samples arrived at the clinical pathology laboratory/ college of veterinary medicine/ Al Muthanna University, the samples were cultured in brain heart infusion broth (BHIB\Himedia, India) and kept at 37˚C for 24 hrs. Later on, a loopful of culture was inoculated on blood agar (B.A\Himedia, India) with 5% sheep blood and MacConkey agar (M.A\ Himedia, India). The identification of the bacterial species was based on observation of their colonial morphology, Gram staining and biochemical characteristics (oxidase, catalase, indol, nitrate, urease, gelatin, simons citrate, motility, TSI, sugar fermentation tests) according to Quinn *et al.,* (2008) (Figure. 3,4, 5).



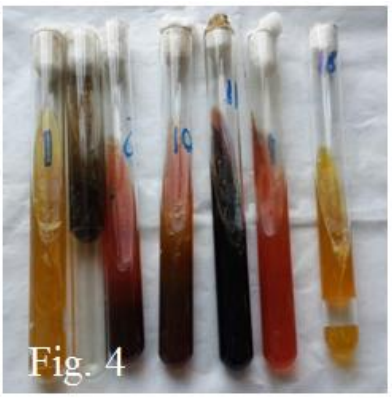


Figure.3: Shows blood agar used for culturing of the samples.
Figure. 4: Shows the TSI used for identification of the bacteria.
Figure. 5: Shows the culture of trachea and lung samples on nutrient agar

All bacterial isolates revealed typical cultural growth, were subjected to Gram's staining and cellular morphology observed with a light microscope (x100). Mixed colonies and Gram-negative bacteria were re-subcultured on both McConkey and blood agar and further incubated aerobically for 24 h. A series of the following biochemical tests: catalase, oxidase, and fermentative/oxidative tests were done for final bacterial identification.

Gross and Histopathological examination

The animals with respiratory signs were examined grossly, and lung lesions in respect to the shape, size, color, and consistency were identified and recorded (Figure. 6). Samples were collected from sheep and goats (lungs and tracheas) from different pathological lesions. Histopathological examination was done at histopathology laboratory at college of Veterinary Medicine/ University of Baghdad. For histopathological studies, all specimens with typical lesions from infected lung, were collected and fixed in 10% neutral buffered formalin saline solution. Tissues were

dehydrated in ethanol using different concentration, cleared in xylene, and embedded in pure white paraffin wax at melting point 56-58°C for preparation of paraffin block. The processed and embedded tissue sections were cut at 3-4 µm with Leica microtome (Leica, Germany). Slides were stained using hematoxylin and eosin (H &E) stain. The slides were examined by light microscope connected with a digital camera.

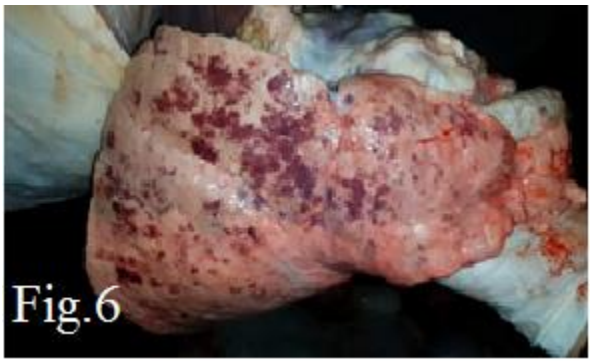


Figure. 6: shows the macroscopical examination of lungs lesions

Molecular Identification of *M. haemolytica*

DNA Extraction

Bacterial cells (up to 1×10^9) were cultured in BHI broth and incubated overnight, the culture was transferred to 1.5 ml micro centrifuge tube and centrifuged at 16,000 g for 1 min. The DNA was extracted using Presto Mini g DNA bacteria Kit according to the manufacturer's instructions (Geneaid, KOBA). The extracted DNA was stored at −20 ° C until use. The DNA concentration was measured by NANODROP-2000 spectrophotometer (Thermo Scientific Inc., USA).

Primers

M. haemolytica oligonucleotide primers were obtained from Integrated DNA Technologies/USA. The primer sequence of *M. haemolytica* 16Sr DNA gene was (F-GCTAACTCCGTGCCAGCAG, R-CGTGGACTACCAG GGTATCTAATC) with size 304 bp (Alexander *et al.,* (2008) and the sequence of 12Sr RNA gene was (F-TAACCCTTGTMCCTTTTGSATRRK, R-AGACTAACTTTTAAAGATACA GTGGG) with size 270 bp (Kumar *et al.,* (2015).

PCR Amplification Analysis

A final volume of 20 µl containing 10 Intron-Master Mix (KOBA), which contains (Taq polymerase, PCR buffer, Gel loading buffer and dNTPs), 2 µl (100 ng of DNA template) and 2 µl of 10 pmol for each primer, was used for PCR amplification. The gene amplification was done with Master cycler (Eppendrof, Germany). Amplified products were separated by agarose gel electrophoresis (1% agaros containing 0.5 mg ethidium bromide in 0.5 × Tris-EDTA electrophoresis buffer) at 90 V/26 mA for 1 h.

A 100 bp DNA ladder (Bioneer, Korea) was used as a molecular size standard, Gel documentation system.

Results

Two hundred and seventy-five (275) animals were examined, including 107 and 163 sheep and goat respectively. The affected animals are represented in (Table. 1). The total number of diseased animals were 104 out of 270 in addition to the five dead animals. The total percentages of the respiratory diseases were 38.51 %. According to sex the number of infected females were 33/ 62 (53.22%) and 19/42 (45/23%) in sheep and goat respectively. Moreover, the number of infected males were 29/62 (46.77%) and 23/42 (54.76%) in sheep and goat respectively. The total number of infected female and male were 52/152 (34.21%) and 52/117(44.44%) respectively.

Table.1: shows the total number of the examined animals and percentages of diseased animals.

Species	Sex		Age	Infected animals			Total number
	Male	Female		Male	Female	Total No.(%)	
Sheep	54	53	5 m – 3 y	29/ 62 (46.77%)	33 /62 (53.22%)	62/ 107 (57.94)	107
Goat	63	100	4 m – 2 y	23/42 (54.76%)	19 /42 (45.23%)	42/ 163 (25.76%)	163
Total	117	153		52 / 117 (44.44%)	52/ 152 (34.21)	104/ 270 (38.51%)	270 + 5 dead animals = 275

Mannheimia haemolytica, *Escherichia coli*, *Pasteurella multocida*, *Klebsiella pneumoniae*، *Staphylococcus aureus* and *Streptococcus pyogenes* were isolated bacteria from the samples of small ruminants with respiratory diseases. Identification of *M. haemolytica* was done depending on colonial morphology on blood agar and McConkey agar. Lung isolates appeared on blood agar as small, gray and rough colonies. While nasal isolates appeared as large, grey and mucoid (smooth) colonies (Figure.7). Lung and nasal isolates showed beta haemolysis on the blood agar after 24 hrs. moreover, the lung isolates appeared as pink pinpoint colonies on McConkey agar, whereas nasal isolate appeared as mucoid pink colonies. While, standard strain of *M. haemolytica* appeared as small, gray and rough colonies. On blood agar haemolysis appeared after 48 h under beneath the colony, and as dark pink pinpoint colonies on McConkey agar. All isolates and standard strain were stained by Gram stain and methylene blue stain, the colonies appeared as G-, coccobacilli or short-rod singly or in pairs, clear bipolarity was appeared by methylene blue stain.

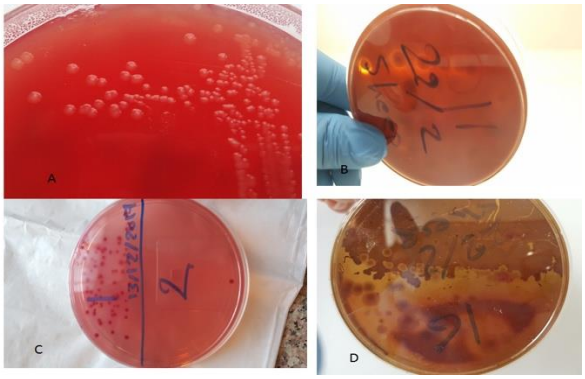


Figure.7: Shows the growth of: A. *Pasteurella multocida*, B & D *Mannheimia. haemolytica* , C. Pink pinpoint colonies of *Mannheimia. haemolytica* on M.A

The examined respiratory system of sheep and goats were revealed various stages of pneumonia and some cases were shown suppurative and exudative pneumonia, congestion (Figure. 8).

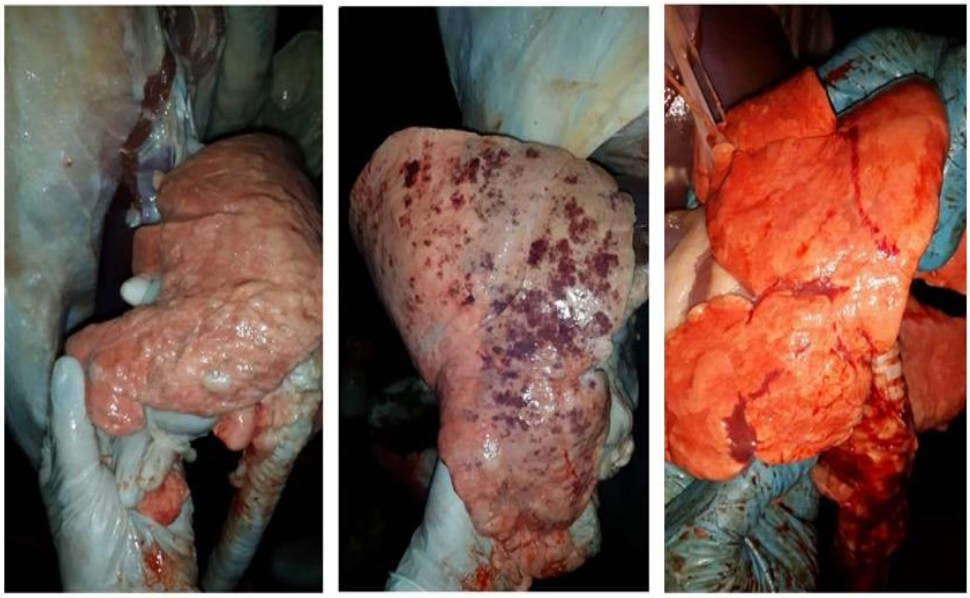


Figure. 8: shows various gross lesions on lung, from left emphysema and nodule, congestions, red hepatisation

Histologically, the following types of pneumonia was seen: suppurative, necrotic and fibrinous bronchopneumonia, bronchointerstitial pneumonia, and pyogranulomatous pneumonia. The lungs diagnosed with bronchopneumonia were characterised by presence of neutrophilic exudates in the alveolar spaces and lumens of the bronchioles and bronchi. In some occasions a mixture of various amounts of cell debris, neutrophils and macrophages were observed in these areas and there were also distended interlobular space, infiltrated with inflammatory cells, distended and collapsed alveoli. While, the lungs with interstitial pneumonia were characterised by interalveolar space infiltrated with predominantly polymorphonuclear cells namely lymphocytes, macrophages and a few neutrophils (Figure. 9).

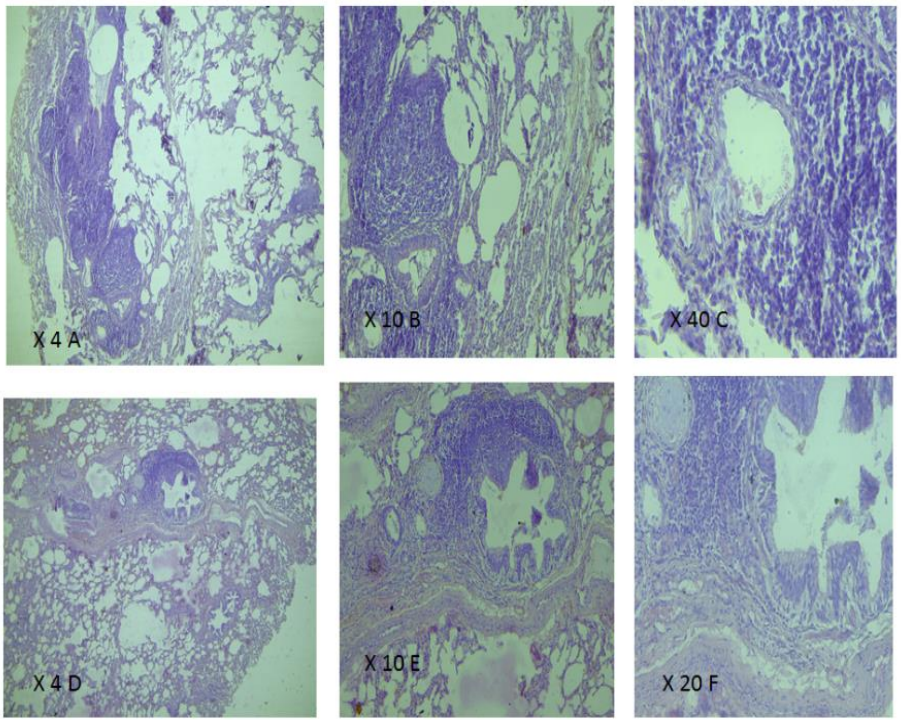


Figure.9: pyogranulomatous pneumonia (A, B & C); bronchointerstitial pneumonia (D, E, F).

M. haemolytica was identified for all isolated strains of by PCR analysis. All isolates were tested to present 16 s rDNA and 12 s rRNA genes. All strains were positive and showed a specific 304 bp and 270 band on agarose gel, no amplification was observed in control negative (10).

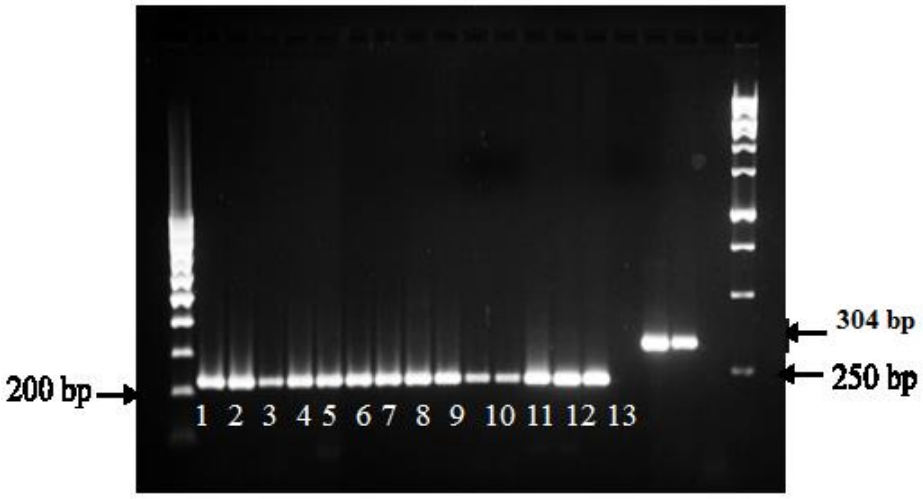


Figure. 11. Agarose gel electrophoresis (1%) of amplified 16 srRNA gene (304 bp) of M haemolytica , stained with ethidum bromide, Lane M: 100 bp ladder (1500 bp) positive M. haemolytica isolates and the standard strain.

Discussion

Respiratory diseases have commonly affected the sheep and goat farms, and affecting individual or groups. It predominantly includes a combination of infectious causes as well as predisposing management factors, significantly leading to potential losses.
The real prevalence of cases of respiratory diseases are higher than the reported percentage that receive by the veterinary Laboratories Agency (only 5·6 % of sheep and goat). In many cases, a possible diagnosis will be made following clinical and on farm postmortem examinations. The results of this study revealed that different bacteria colonized the nasal passage canal of diseased sheep and goats. Previous studies reported similar bacteria from pneumonic lungs in goats and sheep (Lacasta *et al.,* 2008; Garedew *et al.,* 2010). The isolation of *S. aureus* from the nasal passage is compatible with other features obtained from caprine reported in previous study (Tijjani *et al.,* 2012) and ovine lungs (Obasi *et al.,* 2001). Robbins *et al.,* (1981) recorded that *S. aureus* establishes in the upper respiratory tract and is contributed to disease processes only when stress conditions overwhelm. The detection of *E. coli* in nasal passage of goats and sheep is also consistent with features by other authors (Aden *et al.,* 2012). *E. coli*, which is known to be usually harmless in its natural habitat, can cause pulmonary and urogenital tract infection. This may also be related with possible fecal contamination due to the sniffing nature of goats, especially those on heat and during courting before mating. Therefore, the results of this study is compatible with these observations in regard to male goat. The result showed high number of infected goat male 23/42 (54.76%) in compare to sheep male29/62 (46.77%). The constant isolation of *M. haemolytica* from the lungs of different animal species either healthy or having different respiratory signs may point their potential role in infectious pneumopathies (Kamel and Nabil, 2016). *M. haemolytica* was isolated from nasopharynx and tonsils of clinically healthy animals, and serotype A2 is commonly isolated from both sheep and cattle. The organism can also be isolated from lambs and oscillate over time, and this conception has been shown by Ertan, (2006). Moreover, there is a synchronization between the appearance of infections and the occurrence of the organism in the nasopharynx of sheep. However, in calves' nasal passages, the microorganism's flora has been seen to fluctuate in both numbers and species, although *M. haemolytica* can dominate the flora, it can also disappear for weeks at a time (Lopez *et al.,* 1976).
A mechanism owns by *M. haemolytica* that keep it to survive in the upper respiratory tract is still unknown. Moreover, there has also been unsuccessful to culture consistently *M. haemolytica* in swabs collected daily from known colonized animals. The mucociliary clearance mechanism was found to be suppressed by viral infection with or without stress factors. These events allow the proliferation of bacterial commensals in the respiratory tract and also lead to an abrupt shift from commensal to pathogen especially in *M. haemolytica* where serotype 2 shifts to pathogenic serotype 1 (Caswell, 2013). This shift has made *M. haemolytica* to estimate greater prominence in caprine pneumonia. The role of pathogenic bacteria especially *M. haemolytica* is to inhabit the upper respiratory tract of apparently normal sheep and goat that play together with stress factors such as weather, disease and poor management conditions that the animals are continuously exposed. Drug resistance

of some pathogenic bacteria play one of the possible pathogenic role of the normal nasal bacterial flora that has become an abundant, certain, serious problem to both animal and human health care providers. A high level of resistance of *M. haemolytica* to streptomycin had been found. However, the organism was found to be susceptible to ampicillin, oxytetracycline, and chloramphenicol. The prevalence of *M. haemolytica* was also revealed significant differences according to previous studies. It has been reported to range between 8.9% and 96.2% of healthy sheep that carry these organisms in the nasal cavity (AL-Tarazi and Dagnall, 1997). However, the variation is appeared to be caused by several factors including seasonal variation, misidentification, and different isolation techniques.

In the present study, 104 diseased animals out of 270 and five dead animals wete reported. The swabbing of the nasal cavity of sick sheep and goat showed that *M. haemolytica* and other microorganisms were isolated from nasopharyngeal swabs and this result is in agreement with (Gilmour *et al.,* 1974).

Likewise, *M. haemolytica* prevalence was approved to be variant in temperate climates and depending on seasons accompanied by a higher incidence in spring and early summer (Gilmour and Gilmour, 1989). Several researchers found that the frequencies of *Mannheimia* strain isolates were very inconstant. It could sometimes be high and vary according to the origin of infection, like 15.8% in sheep nasal exudates in the United States (Frank, 1982). While, the occurrence in the respiratory system of sheep and goats was 52% for *Mannheimia* and 42% for *P. trehalosi* in Britain, however, in Turkey, the percentage was 8.3% in lungs of sheep (Kirkan and Kaya, 2005). In this study, various types of bacteria were isolated from small ruminants respiratory diseases. These bacteria were *Mannheimia haemolytica, Escherichia coli, Pasteurella multocida, Klebsiella pneumonia, Staphylococcus aureus, and Streptococcus pyogenes.* This result is compatible with previous results reported by another researcher (Hussein *et al.,* 2010; Kifle & Tsegaw, 2012). In ovine nasal exudates, the isolation of *M. haemolytica* was variable according to several researchers such as Blanco *et al.,* (1995) (25%); Pijoan *et al.,* (1999) (35%). Moreover, *M. haemolytica* was the predominate bacteria over *P. trehalosi* in goat and sheep. Even though, *M. haemolytica* was the most frequent isolated serotype in Northern Ireland, *Bali et al.,* (1993) reported that *P. trehalosi* outnumbered *M. haemolytica*. The majority of *M. haemolytica* serotypes were isolated from pneumonia, followed by gangrenous mastitis" blue udder" and septicemia. In Norway, the β-hemolytic *Mannheimia* species were isolated from 24% to 64% of the sheep in four flocks of sheep a total of 26 hemolytic (Poulsen *et al.,* 2006; Biberstein *et al.,* 1970). *M. haemolytica* causes sporadic cases and small outbreaks of acute pneumonia and pleuritis in goat kids (Jubb *et al.,* 1993). At the same time, little is known about the epidemiology of pasteurellosis in goats. Gilmour and Gilmour, (1989) found that *M. haemolytica* was normally associated with pneumonia in cattle and sheep, septicemia in lambs and mastitis in ewes. These findings have subsequently been supported by (Angen *et al.,* 2002; García-Pastor *et al.,* 2009). In this study, various types of bronchopneumonia and pneumonia were observed during the histopathological examination. This result is compatible with previously reported studies in small ruminants in Maiduguri (Tijjani *et al.,* 2012) and Nigeria, (Youssef *et al.,* 2012). Moreover, Ashraf *et al.,* (1986) had reported the incidence and pathology of pneumonia in sheep and goats slaughtered at

Faisalabad, Pakistan. Sheep and goats slaughtered in this study were positive for *E. coli, K. pneumoniae, M. haemolytica, S. pyogenes, S. aureus,* and *P. multocida.* The aerobic bacteria isolated from the pneumonic lungs agreed with the ones isolated by Raji *et al.,* (2000) from ovine and caprine in Zaria, Nigeria and Asaduzzaman *et al.,* (2013) from black Bengal goats in Bangladesh. The identification of microorganisms has been improved by the microbiologists using the phenotypic methods. The *M. haemolytica* was identified in this study by using the phenotypic technique. The phenotypic results of the isolated M. *haemolytica* are in agreement with previous study (Megra *et al.,* 2001), who reported that the M. haemolytica appeared as a large or small, gray and rough or smooth colonies appeared on blood agar with haemolysis that appeared after 24 - 48 hr around or under neath the colonies. Moreover, the colonies appeared as pink to red pinpoint colonies on MacConkey agar, except the nasal mucus isolate appeared as mucoid pink colonies. These results are compatible with (Aschalew,1998). Besides, the characterization of *M. haemolytica* isolates is in agreement with the findings reported by (Homson,1988) and mentioned that the isolate belongs to *M. haemolytica* did not produce indole and grew on MacConkey agar. Conventional biochemical's are routinely used to identify M. haemolytica isolates. The results were in agreement with (Yimer and Asseged, 2007; Collins *et al.,* 1981) that proved the lower suitability of this system for the identification of *M. haemolytica.*

PCR molecular technique has been used for the identification of *M. haemolytica* isolates. In this study, the isolates strain of *M. haemolytica* showed positive results for 16sr RNA gene and corresponds approximately size to 304bp, this result is in agreement with the finding of Alexander *et al.,* (2008) , who used the same primer with successively amplified to 304 bp and sequencing as *M. haemolytica*. Moreover, all isolates also showed positive results for 12sr RNA gene and corresponded to expect size 270 bp. This result is in agreement with (Kumar *et al.,* 2015), who detected *M. haemolytica* directly from lung tissues and bacterial culture by used 12sr RNA gene.

In conclusion, this study approved the incidence of respiratory diseases in small ruminant in Al-Muthanna abattoir. The study also approved the isolation of different microorganisms that might be the cause of respiratory diseases in the small ruminant. Moreover, *M. haemolytica* showed positive results with PCR. The authors, recommend considering PCR as a valuable tool for rapid detection of M. haemolytica in clinical samples from sheep and goats. Also, it offers the opportunity to perform large scale epidemiological studies regarding the role of *M. haemolytica* in clinical cases of pneumonia and other disease manifestations in sheep and other ruminants, thereby providing the basis for effective preventive strategies.

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