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Histopathological, Bacteriological and Molecular study of enzootic respiratory complex of Small Ruminants Slaughtered at Al Muthanna abattoir

Year 5

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Dedication

الاهداء

ألى معلم البشرية وهاديها , ألى النور المبين , ألي رسول الله صلى الله عليه وآلة وسلم...

وألى من رضا الله برضاهما , ألي أمي وأبي ,الى سندي في شدتي ورخائي ...

الى الذين ينظرون الي بعين المحبة و الاحترام , ألي أخوتي و أخواتي

ألى من تابعني ونصحني ووجهني حتى اتممت بحثي , الى أستاذتي الفاضلة ...

ألى من هم لولاهم لما كنا نكمل حياتنا ...

الحشد الشعبي المقدس والجيش العراقي البطل ...

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نتقدم بالشكر والجزيل لأستاذتنا في جامعة المثنى \ كلية الطب البيطري

لجهودهم المتميزة خلال سنين الدراسة ولتوجيهاتهم لنا اثناء كتابة البحث فجزاهم الله خيراً عن العلم وآهله ...

كما نقدم اسمى آيات الشكر و الامتنان و المحبة و التقدير الى أستاذتنا المشرفة الدكتورة (كريمة عاكول الصالحي) لما احاطتنا به من رعاية علمية , وما بذلته من جهد ووقت ثمين , ومنحتنا ثقه و إيمان بالعمل ...

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وألى جميع استاذتنا في جامعة المثنى \ كلية الطب البيطري .. ونتقدم بالشكر الجزيل لكل من مد يد العون من التوجيه وتوفير المصادر ...

الى كل هؤلاء شكرا جزيلا

الباحثون

Abstract

Small ruminants play an important role in the nutrition and income of people around the world. They serve as source of meat, milk, skin and wool. Small ruminants contribute significantly to the economy of farmers in the Iraq. The mass production of small ruminant in the country is constrained by disease, inadequate nutrition, poor genetic resources of the local stock, marketing, social factors, structural constraints. Diseases of respiratory system contributes to high morbidity and mortality rate in the sheep and goat in Iraq.This study intends to study histopathological, bacteriological and molecular study of enzootic respiratory complex of small ruminant that slaughtered in Al Muthanna province. This study was conducted in Samawah city / Al Muthanna Government 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. Before slaughtering all animals were examined for any signs of respiratory diseases. One hundred four nasal swabs were collected from nasopharyngeal area of sheep and goats that showed nasal discharge. The nasal swab from each diseases sheep and goat was analyzed using standard methods. Tissue samples were collected from animals and processed routinely for histopathological investigation. Molecular Identification was done for isolated *Mannheimia haemolytica.* The results of this study showed that the total number of diseased animals was 104 out of 270, in addition to the five dead animals. The percentages of the respiratory diseases were 38.51 % including 29/ 62 (46.77 %) and 33/62 (53. 22%) for male and female respectively in sheep and totally 62/107 (57. 94%). While, the percentages of respiratory diseases in goat were 23/42 (54.76%) and 19 /42 (45.23%), and totally 42/ 163 (25.76%).The aerobic isolated bacteria associated with respiratory in small ruminants were *Mannheimia haemolytica, Escherichia coli, Pasteurella multocida, Klebsiella pneumoniae and Staphylococcus aureus and Streptococcus pyogenes*. The gross lesions observed were majorly, suppurative pneumonia, exudative pneumonia, congestion, and various stages of pneumonia. Histologically, the following types of pneumonia was seen : suppurative bronchopneumonia necrotic bronchopneumonia, fibrinous bronchopneumonia, bronchointerstitial pneumonia, and pyogranulomatous pneumonia. The lungs diagnosed with bronchopneumonia were characterised by neutrophilic exudates were present in the alveolar spaces and lumens of the bronchioles and bronchi, and in some occasions a mixture of various amounts of cell debris, neutrophils and macrophages were observed in these areas and there are also distended interlobular space, infiltrated with inflammatory cells, distended alveoli 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. Identification M. haemolytica was confirmed for all strains of by PCR analysis. The test was performed, all isolates were tested to present 16 s rDNA and 12 s rRNA genes. Hereafter, all strains were positive for all isolated bacteria and 12 s primers, they showed a specific 304 bp and 270 band respectively on agarose gel, no amplification was observed in control negative. 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 microorgaisms that might be the cause of respiratory diseases in this study, moreover, M. haemolytica showed a positive results with PCR. The author, recommoned to consider PCR as a valuable tool for rapid detection of M. haemolytica in clinical samples from sheep and goats. In addition, 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.

الخلاصة

تلعب المجترات الصغيرة الاغنام والماعز دوراً هاماً في تغذية ودخل الناس في جميع أنحاء العالم. وتشكل بمثابة مصدر للحليب واللحوم والجلد والصوف . تساهم المجترات الصغيرة مساهمة كبيرة في اقتصاد المزارعين في العراق. إن الإنتاج الضخم للمجترات الصغيرة في البلد مقيد بالمرض ، وعدم كفاية التغذية ، وضعف الصفات الوراثية للحيوانات المحلية ، والتسويق ، والعوامل الاجتماعية ، والقيود الاخرى . تساهم أمراض الجهاز التنفسي في ارتفاع معدلات الحيوانات المريضة والهلاكات في الأغنام والماعز في العراق. تهدف هذه الدراسة إلى دراسة التغيرات النسيجية والجرثومية والجزيئية لمرض الجهاز التنفسي المركب للمجترات الصغيرة التي ذبحت في مجزرة محافظة المثنى. أجريت هذه الدراسة في مدينة السماوة / محافظة المثنى خلال فترة بدأت من أكتوبر / تشرين الأول 2017 إلى مارس / آذار 2018. تم إجراء دراسة استقصائية مقطعية على الأغنام والماعز المذبوحة في مسلخ المثنى. تم فحص جميع الحيوانات قبل الذبح بحثًا عن أي علامات لأمراض الجهاز التنفسي. تم جمع مائة أربعة مسحات الأنف من منطقة البلعوم من الأغنام والماعز التي أظهرت إفرازات الأنف. تم تحليل مسحة الأنف من كل من الأغنام والماعز باستخدام طرق قياسية. تم جمع عينات الأنسجة من الحيوانات ومعالجتها بشكل روتيني للفحص النسيجية. وقد تم التاكد من هوية جرثومة المعزولة *Mannheimia haemolytica*

أظهرت نتائج هذه الدراسة أن العدد الإجمالي للحيوانات المريضة كان 104 من أصل 270 ، بالإضافة إلى الحيوانات الميتة الخمسة. بلغت نسبة أمراض الجهاز التنفسي 38.51٪ بما في ذلك 62/29 (46.77٪) و 62/33 (53. 22٪) للذكور والإناث على التوالي في الأغنام و 107/62 (57. 94٪). في حين كانت النسب المئوية لأمراض الجهاز التنفسي في الماعز 42/23 (54.76٪) و 42/19 (45.23٪) و 163/42 (25.76٪) ، وكانت البكتيريا الهوائية المعزولة المرتبطة بالجهاز التنفسي في المجترات الصغيرة هي

Mannheimia haemolytica ،Escherichia coli ،Pasteurella multocida ،Klebsiella pneumoniae and Staphylococcus aureus and Streptococcus pyogenes.

عيانيا كانت الإصابات الشديدة التي لوحظت هي الالتهاب الرئوي بشكل رئيسي ، والالتهاب الرئوي التقيحي ، والالتهاب الرئوي النافر ، والاحتقان ، ومراحل مختلفة من الالتهاب الرئوي. من الناحية النسيجية ، شوهدت الأنواع التالية من الالتهاب ، القصبي الفبريني ، التهاب الرئة necrotic bronchopneumonia الرئوي: التهاب القصبات القصبي القيحي pyogranulomatous and bronchointerititial
، وفي بعض الحالات لوحظ وجود خليط من كميات مختلفة من حطام الخلايا والعدلات والبلاعم في هذه المناطق
وتم اختبار جميع عزلات ال *Mannheimia haemolytica*باستخدام فحص البي سي ار للتاكد من هويتها الجينية.

حيث اثبتت ايجابيتها لوجود 16 s rDNA and 12 s rRNA genes.
. في الخلاصة ، اثبتت هذه الدراسة حدوث أمراض الجهاز التنفسي في المجترات الصغيرة في مسلخ المثنى. كما واثبتت الدراسة على عزل الجراثيم المختلفة التي قد تكون السبب في أمراض الجهاز التنفسي في هذه الدراسة ، وعلاوة على ذلك ظهر بان الفحص الجزيئي قادر على تحديد التركيب الجيني للمسببات المرضية

. بالإضافة إلى ذلك ، فإنه يوفر الفرصة لإجراء دراسات وبائية واسعة النطاق فيما يتعلق بدور هيموليتيكا السريرية للالتهاب الرئوي وغيرها من مظاهر المرض في الأغنام والمجترات الأخرى ، وبالتالي توفير أساس لاستراتيجيات وقائية فعالة.

Contents

Introduction

Small ruminants are valuable possessions for the Mediterranean, African, and Southeast Asian countries with the potential for providing meat, milk, and wool. These animals are highly susceptible to respiratory diseases, which account for almost 50% mortality amongst them. Irrespective of the etiology, the infectious respiratory diseases of sheep and goats contribute to 5.6 percent of the total diseases of small ruminants. The infectious respiratory disorders are classified into two groups: the diseases of upper respiratory tract including sinusitis caused by the larvae of parasites, nasal foreign bodies, gaseous irritation, and enzootic nasal tumors and the diseases of lower respiratory tract comprising mainly pneumonia. Often these are of infectious origin (bacterial, viral, or fungal). However, the role of the environmental pollutants, toxicants, and mechanical induction of respiratory distress may also be the cause of these abnormal conditions. Depending upon the environmental, physiological, and etiological factors, respiratory conditions might be acute, chronic, and/or progressive in nature.

To overcome such important disease conditions, information on their identification, prevention, cure, and control can improve the economic status and sustainability of holders of small ruminants. Thus an early, rapid, and specific diagnosis of such diseases holds great importance to reduce 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 disease conditions with specificity and sensitivity. Similarly, molecular diagnostic assays along with microsatellites comprehensively assist in diagnosis as well as treatment and epidemiological studies. It is necessary to take appropriate prevention protocols and devising suitable control strategies to overcome such important respiratory diseases, thus alleviating the economic losses. A number of pathogenic microorganisms have been implicated in the development of respiratory diseases but the importance of environmental factors in the initiation and progress of the disease can never be overlooked. These environmental factors irritate the respiratory track producing stress in the microenvironment causing a decline in the immune status of the small ruminants and thereby assisting bacterial, viral, and parasitic infections in breaking down the tissue defense barriers. Environmental pollutants cause acute or chronic reactions as they deposit on the alveolar surface, which are characterized by inflammation or fibrosis and the exhibition 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. Moreover, the environmental scenario is never static; new compounds are introduced daily making a precise evaluation of the disease burden almost impossible.

It is assume a uniquely important position in live stock production. Unlike cattle, small ruminants are capable of remarkable adaptability to diverse environment 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 national Iraqi 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). They supply a high percentages of all domestic meat consumption and generate cash income from exports of meat, mainly as live animals and skin. Therefore, an increase in small ruminant production is needed both to maintain self-sufficiency and to increase export earnings. 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 shortage of high level of trained man power (5). Of these, multifactorial infectious diseases of small ruminants cause substantial loss through morbidity and mortality (6). Thomson (7) stated that all diseases, those affecting the respiratory system are generally the most important in every species of domestic animals. Bacterial infection of the respiratory tract may be primary, occurring in healthy individuals or secondary to a large number of conditions which depress resistance. Secondary bacterial infection occur especially when the local resistance of the respiratory mucosa is lowered and bacterial growing in the nose and throat extends down wards, usually giving a mixed infection (8).

Aims of study

No studies have been done on bacteriological, histopathological and molecular examination of pneumonic lung of small ruminants in Al Muthanna province. Consequently, this study intends with objectives of isolation and identifying bacteria involved in enzootic respiratory disease of small ruminants and studying the histopathological changes of the affected lungs. Moreover, to characterize the isolated *Mannheimia haemolytica* using (PCR) molecular technique.

Review of literature

Small ruminants particularly sheep and goats contribute significantly to the economy of farmers in Mediterranean as well as African and Southeast Asian countries. These small ruminants are valuable assets because of their significant contribution to meat, milk, and wool production, and potential to replicate and grow rapidly. The great Indian leader and freedom fighter M. K. Gandhi "father of the nation" designated goats as "poor man's cow," emphasizing the importance of small ruminants in poor countries. In Iraq, sheep and goats play a vital role in the economy of a high populations. To make this small ruminant based economy viable and sustainable, development of techniques for early and accurate diagnosis holds prime importance. Respiratory diseases of small ruminants are multifactorial (1) and there are multiple etiological agents responsible for the respiratory disease complex. Out of them, bacterial diseases have drawn attention due to variable clinical manifestations, severity of diseases, and reemergence of strains resistant to a number of chemotherapeutic agents (2). However, sheep and goat suffer from numerous viral diseases, namely, foot-and-mouth disease, bluetongue disease, maedi-visna, orf, Tick-borne encephalomyelitis, peste des petits ruminants, sheep pox, and goat pox, as well as bacterial diseases, namely, blackleg, foot rot, caprine pleuropneumonia, contagious bovine pleuropneumonia, Pasteurellosis, mycoplasmosis, streptococcal infections, chlamydiosis, haemophilosis, Johne's disease, listeriosis, and fleece rot (3-10).

The respiratory diseases represent 5.6 per cent of all these diseases in small ruminants (11). Small ruminants are especially sensitive to respiratory infections, namely, viruses, bacteria, and fungi, mostly as a result of deficient management practices that make these animals more susceptible to infectious agents. The tendency of these animals to huddle and group rearing practices further predispose small ruminants to infectious and contagious diseases (6, 9). In both sheep and goat flocks, respiratory diseases may be encountered affecting individuals or groups, resulting in poor live weight gain and high rate of mortality (5). This causes considerable financial losses to shepherds and goat keepers in the form of decreased meat, milk, and wool production along with reduced number of offspring. Adverse weather conditions leading to stress often contribute to onset and progression of such diseases. The condition becomes adverse when bacterial as well as viral infections are combined particularly under adverse weather conditions (1). Moreover, under stress, immunocompromised, pregnant, lactating, and older animals easily fall prey to respiratory habitats, namely, *Streptococcus pneumoniae*, *Mannheimia haemolytica*, *Bordetella parapertussis*, *Mycoplasma* species, *Arcanobacterium pyogenes*, and *Pasteurella species* (2, 4, 7-9, 12,13). Such infections pose a major obstacle to the intensive rearing of sheep and goat and diseases like PPR, bluetongue, and ovine pulmonary adenomatosis (Jaagsiekte) adversely affect international trade (2,9,10, 13), ultimately hampering the economy.

2. Respiratory Diseases of Small Ruminants

Depending upon the involvement of etiological agent, the infectious respiratory diseases of small ruminants can be categorized as follows(9,14):

1. bacterial: Pasteurellosis, Ovine progressive pneumonia, mycoplasmosis, enzootic pneumonia, and caseous lymphadenitis,
2. viral: PPR, parainfluenza, caprine arthritis encephalitis virus, and bluetongue,
3. fungal: fungal pneumonia,
4. parasitic: nasal myiasis and verminous pneumonia,
5. others: enzootic nasal tumors and ovine pulmonary adenomatosis (Jaagsiekte).

Many times due to environmental stress, immunosuppression, and deficient managemental practices, secondary invaders more severely affect the diseased individuals; moreover, mixed infections with multiple aetiology are also common phenomena (5, 8, 13, 15). These conditions involve respiratory tract as primary target and lesions remain confined to either upper or lower respiratory tract (7, 16). Thus, these diseases can be grouped as follows(5,8, 14, 17).

1. Diseases of upper respiratory tract, namely, nasal myiasis and enzootic nasal tumors, mainly remain confined to sinus, nostrils, and nasal cavity. Various tumors like nasal polyps (adenopapillomas), squamous cell carcinomas, adenocarcinomas, lymphosarcomas, and adenomas are common in upper respiratory tracts of sheep and goats. However, the incidence rate is very low and only sporadic cases are reported.
2. Diseases of lower respiratory tract, namely, PPR, parainfluenza, Pasteurellosis, Ovine progressive pneumonia, mycoplasmosis, caprine arthritis encephalitis virus, caseous lymphadenitis, verminous pneumonia, and many others which involve lungs and lesions, are observed in alveoli and bronchioles.

Depending upon the severity of the diseases and physical status of the infected animals, high morbidity and mortality can be recorded in animals of all age groups. These diseases alone or in combination with other associated conditions may have acute or chronic onset and are a significant cause of losses to the sheep industry (3, 10). Thus, the respiratory diseases can also be classified on the basis of onset and duration of disease as mentioned below (3, 9, 14, 18):

1. acute: bluetongue, PPR, Pasteurellosis, and parainfluenza,
2. chronic: mycoplasmosis, verminous pneumonia, nasal myiasis, and enzootic nasal tumors,

3. progressive: Ovine progressive pneumonia, caprine arthritis encephalitis virus, caseous lymphadenitis, and pulmonary adenomatosis.

3. Need of Advanced Diagnostic Approaches

The potential losses due to respiratory diseases can be minimized by sound diagnostic approach along with sound management program (15). Any kind of compromise with the diagnostic and management approach would severely affect the health status of the flock (19). Early, rapid, and effective diagnosis of the respiratory diseases in small ruminants is a challenge due to limited laboratory resources in Iraq and Southeast Asian countries where a large small ruminant population gets decimated due to respiratory disease outbreaks(15,16). Conventional methods of diagnosis may be available more frequently but they usually take longer to yield results, and also their specificity and sensitivity may not be up to the mark. In recent past, many advanced, rapid, sensitive, and specific serological and molecular tests have been developed. These diagnostic methods have supplanted the conventional diagnostic procedures owing to their speed, sensitivity, specificity, and applicability even without isolation of etiological agent (20, 21).

In present scenario of globalization and regulations related to international trades, continuous monitoring of enlisted diseases is mandatory and for that sampling, isolation, and confirmation processes are very tedious(22, 23). In such scenario, the rapid and specific detection of antibodies to the respiratory pathogens is now possible by the advancement in serological testing. Availability of better serological tests including ELISAs and monoclonal antibodies has enabled detection of antibodies to these infectious agents (namely, bacteria, viruses, and fungi) with more rapidity as well as specificity (24). Moreover, due to advancement in the polymerase chain reaction (PCR) technology, there has been enormous improvement in the diagnosis of respiratory diseases of small ruminants (25). Recent advances in biotechnology and molecular biology have led to the development of a variety of diagnostic assays, namely, PCR, RT-PCR, PCR-ELISA, RAPD, AFLP, RFLP, real-time PCR, quantitative PCR, multiplex PCR, LAMP, microsatellites, gene sequencing, and phylogenetic analysis, which not only help in identification but also assist in molecular characterization of various pathogens (20, 22, 37). Various conventional diagnostic tests, namely, isolation, postmortem finding, and gross clinical examinations along with modernized serological and molecular tests, are enlisted in Figure 1.

Figure. 1 :Diagnosis of infectious respiratory diseases of small ruminants.

Advances in diagnostic tools and assays help strengthening the surveillance and monitoring systems of animal diseases. The latest advances in molecular techniques have assisted in the rapid and confirmatory diagnosis of the diseases and epidemiological studies to formulate appropriate and timely prevention, treatment, and control measures, and alleviation of economic losses to animal producers (1, 7, 13, 22, 23).

4. Advances in Diagnosis of Respiratory Diseases of Small Ruminants

For the prevention and control of fatal infectious respiratory diseases of small ruminants, various diagnostic strategies are adopted worldwide. The diagnostic tests as well as procedures adopted in different parts of world incorporate combination of conventional and advanced diagnostic tests. However, the initial suggestive diagnosis involves the observation of clinical signs and postmortem findings followed by serological and molecular methods for the confirmation of

etiological agents. Common infectious respiratory diseases of small ruminants, clinical signs, postmortem findings, and diagnostic tests are compiled in Table.1.

4.1. Peste des Petits Ruminants (PPR)

Peste des petits ruminants (PPR) is an acute and highly contagious viral disease of small ruminants and in particular of goats, popularly known as goat plague [38, 39]. Transmission of the disease takes place by direct contact with the secretions or excretions from the infected animals to healthy ones, which are in close contact. Clinically, PPR is characterized by pyrexia, ocular and nasal discharges, erosive stomatitis, and diarrhea [38, 40]. The postmortem findings are limited mainly to the alimentary tract that consists of erosive stomatitis (extensive in nature) as well as hemorrhagic gastroenteritis. Often, streaks of congestion may be found along the mucosal folds that result in the characteristic appearance of "zebra-strip" [131, 132]. The morbidity and mortality rates of PPR can be as high as 100% and over 90%, respectively [39, 40].

The various serological tests applied in the PPR detection include agar gel immunodiffusion, virus neutralization, complement fixation, haemagglutination inhibition, and competitive ELISA assays. Conventional serological tests like complement fixation or haemagglutination inhibition cannot differentiate between PPR and Rinderpest (RP). However, haemagglutination inhibition (HI) can be used quantitatively for the measurement of PPRV antibodies in suspension. Titration of the PPRV antigen can be done by the use of both haemagglutination (HA) and HI tests [39–41]. Peste des petits ruminant's virus (PPRV) can be differentiated from Rinderpest (RP) by virus neutralization and competitive ELISA assays. Competitive ELISA can be a better choice for detection of antibody to PPR because of its high specificity of diagnosis [40]. A rapid as well as sensitive and virus-specific test for detection of PPRV antigen is immunocapture ELISA that can cause differentiation of RP and PPR. It has got higher sensitivity than routinely used agar gel immunodiffusion test (36, 42, 133).

Table.1: Common infectious respiratory diseases of small ruminants

Sl. no.	Name of condition	Etiological agents	Affected species	Clinical signs	PM findings	Diagnostic tests	References
1	Peste des petits ruminants (PPR)	*Morbillivirus* (family Paramyxoviridae)	Goats and sheep	Mucopurulent nasal and ocular discharges, necrotizing and erosive stomatitis, enteritis, and pneumonia	Congestion of mucosa of respiratory tract, exudates in tract, hardening of lungs mainly in anterior lobes, congestion hemorrhages, and erosion in intestinal mucosa	HA, HI, ELISA, PCR-ELISA, RT-PCR, real-time PCR LAMP	[29, 30, 37–43]
2	Bluetongue	*Orbivirus* (family Reoviridae)	Goats and sheep	Swelling of the lips and tongue and typical blue discoloration of tongue, though this sign is confined to a minority of the animals. Nasal symptoms may be prominent, with nasal discharge and stertorous respiration	In sheep-oedematous face and ears, with dry, crusty exudate on the nostrils, hyperemic coronary bands of hooves, petechial or ecchymotic hemorrhages may be present and extend down the horn. Petechiae, ulcers, and erosions in the oral cavity, particularly on the tongue and dental pad. The oral mucous membranes may be necrotic or cyanotic. The nasal mucosa and pharynx may be edematous or cyanotic, and the trachea hyperemic and congested. Froth is sometimes seen in the trachea	AGID, RNA-PAGE, FIT, RT-PCR, ELISA, and real-time PCR	[44–58]
3	Parainfluenza	Paramyxovirus	Sheep and goats	Mostly associated with enzootic pneumonia	Generally followed by bacterial infection mainly secondary *Mannheimia* infection	Direct immunofluorescence, ELISA, RT-PCR, real-time PCR, and multiplex PCR	[11, 18, 59–65]
4	Caprine arthritis encephalitis virus	Retrovirus	Neurologic disease in kids and arthritis in adults	Lymphocytic mastitis and hard firm udders along with chronic wasting	Signs of secondary bacterial infection: thickening of interalveolar septa and lymphoid hyperplasia, chronic interstitial pneumonia	TaqMan quantitative PCR (qPCR)	[18, 66–71]
5	Ovine progressive pneumonia (maedi-visna)	Oncogenic retrovirus of subfamily lentiviridae	Sheep	Chronic emaciation, weakness, dyspnea, lymphoproliferative pneumonia, meningeal arteritis with encephalitis, nonsuppurative arthritis, and lymphocytic mastitis	Characteristic firm lungs with greyish to brown discoloration, thickening of interalveolar septa, and lymphoid hyperplasia	Immunohistochemical (IHC), cELISA, AGID, IF and real-time quantitative PCR (qPCR)	[18, 72–76]

Sl. no.	Name of condition	Etiological agents	Affected species	Clinical signs	PM findings	Diagnostic tests	References
6	Enzootic nasal tumors, ovine pulmonary adenomatosis (Jaagsiekte)	Retrovirus	Sheep and goats	Signs of inspiratory dyspnea along with serumucoid nasal discharge	Presence of uni- or bilateral tumor growth; firm, hard, grey colored lungs; lungs sink in water, and bronchi are found filled with white frothy fluid	ELISA, RT-PCR, real-time PCR, IHC, and nested PCR	[77–83]
7	Enzootic pneumonia (Pasteurellosis, shipping fever, and hemorrhagic septicemia)	*M. haemolytica* and *Bibersteinia trehalosi* (*Pasteurella trehalosi*)	Sheep	Dyspnea, pyrexia, dullness, depression, mucopurulent nasal discharge, oculonasal blood, and tinged discharge	Semifibrinous fluid in lungs with fibrinous adhesions leading to consolidation of lungs	Counterimmunoelectrophoresis, ELISA, PCR, multiplex PCR, IHC, ISH, AFLP, AP-PCR, DNA fingerprinting, and Southern blot	[14, 84–96]
8		*M. haemolytica* and *P. multocida*	Goats				
9	Caseous lymphadenitis	*Corynebacterium pseudotuberculosis*	Sheep and goats	Chronic emaciation, dyspnea, exercise intolerance, dullness, forced full coughing, and weight loss	Enlargement of lymph nodes with greenish colored pus	Haemagglutination test, Counterimmunoelectrophoresis, IFN-γ ELISA, and PCR	[14, 97–103]
10	Mycoplasma	*M. ovipneumoniae*, *M. capricolum*, *M. mycoides* subsp. *mycoides*, and *M. agalactiae*	Sheep and goats, kids may develop encephalitis	Anorexia, pyrexia, painful breathing, coughing, and sneezing	Peribronchiolar lymphocytic infiltrations are observed with diffused nonsuppurative pleuritis	Immunoblotting, immunobinding assay, growth inhibition, PCR-RFLP, and multiplex real-time PCR	[7, 38, 104–119]
11	Nasal myiasis	*Oestrus ovis*	Sheep	Stamping of feet, efforts to hide nose, difficult breathing, and heavy sound in respiration	Swollen nasal membranes, plugged nostrils, and upper respiratory tract occluded with serofibrinous discharge	Double immunodiffusion (DD), indirect haemagglutination (IH) tests, ELISA, and PCR PCR-RFLP	[120–125]
12	Verminous pneumonia	*Dictyocaulus filaria*, *Protostrongylus rufescens*, and *Muellerius capillaris*	Young animals in the age group of 2–18 months	Pyrexia, coughing, rapid and painful breathing, nasal discharge, and emaciation with retarded growth	Presence of parasites and caseous exudates in lungs	ELISA and PCR	[14, 126–130]

There has been a substantial improvement in the techniques to detect the nucleic acids of PPRV. PCR assays are now considered as powerful as well as novel means of detection and quantification of the nucleic acids of PPR virus in various types of clinical samples. But unfortunately, no single assay can detect all the lineages of the virus. Companion tests can be developed by manipulation of the PPRV gene and insertion of either positive or negative markers (25, 29, 30). The nucleoprotein based RT-PCR, which is based on nucleoprotein (N) genes, has been standardized recently. Instead of analysis of the amplified product by means of agarose gel electrophoresis, its detection is done on a plate by ELISA using labeled probe. The sensitivity of

this RT-PCR ELISA is ten times higher than the classical RT-PCR. With the aid of quantitative real-time RT-PCR, there has been significant improvement in the diagnosis of PPR (29,30). This minimizes the risk of contamination. There is also description of applying nucleic acid amplification for the diagnosis of PPR. The sensitivity of this assay is similar to that of PCR but its simplicity in implementation, as the results can be read by naked eye, and rapidity make it suitable for practical application (32, 34). Use of LAMP significantly reduced the processing time of sample and final outcome (21). Similarly, LAMP assay based on conserved region of "N" gene of PPR virus has been documented for rapid and specific detection of PPR virus from clinical samples. The assay was found 100–1000 times superior to PCR and s-ELISA (43). Synthetic peptide and multiple antigenic peptide based antigen has been employed in ELISA for detection of PPR virus antibodies. A PCR-ELISA based on N gene has been standardized in order to detect PPR virus thereby yielding a product (which is labeled with digoxigenin) that comprises a sequence from N gene of the PPRV. The assay has been found to be more sensitive than sandwich ELISA in order to detect the virus in both early and late phases of the disease. For differential diagnosis of PPRV from Rinderpest virus, also the assay has been found to be useful (27). A one-step multiplex RT-PCR (single tube) has been standardized for amplification of specific fragments of the N as well as M genes of PPR virus. For detection of the virus directly from clinical field samples, the RT-PCR is conducted by the use of purified viral RNA. The assay is easier than the two-step assay as it is time saving requiring only one buffer for both reverse transcription and PCR (29). RT-PCR based on F gene has shown a low sensitivity as well as specificity along with moderate agreement as compared to sandwich ELISA (36). By the use of one-step Brilliant SYBR Green Kit, a sensitive as well as rapid single step real-time RT-PCR has been standardized for detection and semiquantitation of PPRV by the use of primers specific to viral RNA and matrix protein gene. They have been compared with conventional RT-PCR as well as Taqman RT-PCR. It has been found that the assay is more rapid as well as sensitive than TaqMan and the conventional RT-PCR in order to detect nucleic acid of PPRV from the clinical samples of sheep as well as goat, which are suspected for PPR. As an alternative test to the various diagnostic assays that already exist, SYBR Green RT-PCR has been found to be a successful tool thereby helping in rapid clinical diagnosis with advantage of reducing contamination risk (30,37).

4.2. Bluetongue

Bluetongue (BT) is one of the important infectious diseases of domestic and wild ruminants. It is caused by bluetongue virus (BTV) of genus *Orbivirus* and family Reoviridae. The disease, transmitted by *Culicoides*(biting midges), was first reported in India in 1964 (134). India has

significant populations of domestic and wild ruminants, which are known to be susceptible to BTV infection. Several exotic breeds of sheep were introduced into the country between 1960 and 1970 for genetic improvement of the national flock by crossbreeding with native breeds (135). This increase in the susceptible population, along with favorable climatic conditions, appears to have led to the establishment of BTV in the country (135, 136). The disease has an incubation period of 5–20 days with the development of symptoms within a month. There is low mortality rate but in susceptible breeds of sheep the mortality may be high (136). Asymptomatic infection is usually observed in cattle as well as goats and wild ruminants despite the high level of virus in the blood. Exception is red deer in which the disease may be as acute as in sheep (137). The development in diagnostic technologies has confirmed over the past that BTV is now widely spread in several parts of India(136,138). Traditionally, the diagnosis of BTV is primarily based on clinical signs and symptoms. However, differential diagnosis with some of the diseases such as contagious ecthyma, foot and mouth disease (FMD), vesicular stomatitis, malignant catarrhal fever (MCF), bovine virus diarrhea (BVD), infectious bovine rhinotracheitis (IBR), parainfluenza-3 infection, and sheep pox should be done (135, 136, 139). The confirmatory diagnosis may be done either through virus isolation or through serological test. The virus isolation is performed in embryonated chicken eggs, in cell culture (BHK-21 or Vero cell line), or occasionally in sheep (139). The virus is serotyped either by virus neutralization tests such as plaque reduction, plaque inhibition, Microtiter neutralization, and Fluorescence inhibition test (FIT) or through reverse-transcription polymerase chain reaction (RT-PCR) (a prescribed test for international trade) (50, 51). A highly sensitive silver staining method of RNA-polyacrylamide gel electrophoresis (RNA-PAGE) of bluetongue virus was developed recently (48). Various serological tests such as complement fixation test (now largely replaced by the AGID test), agar gel immunodiffusion, and competitive enzyme-linked immunosorbent assay (both are prescribed test for international trade) are used for serological characterization of BTV. Recently, novel Indian isolates of BTV 21 were detected employing real-time PCR assay (44). The complete genome sequence of BTV serotype 16 of goat origin from India has also been carried out (140). Similarly, the complete genome sequences of BTV22 and reassortment strain of BTV 2, 3, 16, and 23 from India have been carried out recently (45,48,52). Analyses of the nucleotide sequence as well as phylogenetic comparisons of genome segment 2 that encodes outer-capsid protein VP2 help in creation of segment-2 database (53). Such database is used for developing rapid as well as reliable typing assay based on RT-PCR (50, 51, 54). Testing of multiple primer pairs has also been done that provides an identification of serotype initially by amplifying a cDNA product of the expected size. Confirmation of serotype has been done by sequencing of the cDNA amplicons and subsequently phylogenetic analysis is done for comparing with reference strains

that are previously characterized(52,54). The RT-PCR assay provides a rapid as well as sensitive and reliable method to identify and differentiate all the serotypes of BTV (45, 50, 51, 55–58).

4.3. Parainfluenza

Parainfluenza is mainly characterized at necropsy by purulent bronchopneumonia (focal) along with moderate to severe pulmonary congestion. Histopathological analysis has revealed the presence of acute and severe as well as diffuse necrotizing and fibrinous or suppurative bronchopneumonia. There is also a presence of diffuse congestion as well as pulmonary edema (61). As a diagnostic method, comparison of enzyme immunoassay has been done with complement fixation test (CFT). The cross-reactivity of the viruses can be detected by the application of such tests (59). Parainfluenza is a viral infection of the lower respiratory tract causing an enormous burden of disease in small ruminants. Direct immunofluorescence technique along with cross-neutralization tests is required for antigenic analysis of the parainfluenza virus isolates. For detection of the virus associated with it, new diagnostic test like multiplex PCR has got enormous advantages mainly because of its specificity (17). Real-time PCR (RT-PCR) is a useful molecular tool for detection of parainfluenza virus type 3 (Pi3) from ribonucleic acid (RNA) samples from cells of the lungs from the slaughtered animals. This is followed by sequencing as well as restriction enzyme patterns of the fragment amplified of the F gene which confers confirmation of the distinctness of the isolates. Availability of suitable PCR primers allows detection of the ovine virus specifically (62). Phylogenetic analysis of the amino acid as well as the nucleotide sequences is also equally important (60). In some of the instances, it has been seen that the in-house RT-PCR methods cannot yield expected products for which the nucleotide sequence analysis has been initiated (63). Multiplex RT-PCR can help distinguish parainfluenza viruses from other respiratory virus like adenovirus (64). Nucleic acid sequence based amplification (NASBA) has been developed for which primers as well as probes have been selected from the haemagglutinin-neuraminidase (HN) gene as well as from the phosphoprotein (P) of the parainfluenza virus (61,65).

4.4. Caprine Arthritis Encephalitis Virus

Caprine arthritis encephalitis virus (CAEV) is a member of the lentivirus family (in small ruminants) leading to chronic disease of the joints and rarely encephalitis in goat kids under the age of six months. The virus is in close intimation with white blood cells. Thus, any kinds of body secretions containing blood cells are potential sources for virus spread to other animals in the herd (141, 142). In goats ,in order to detect caprine arthritis encephalitis virus (CAEV), serological tests or cell cultures are mainly used. Besides, PCR has also been developed for

detection of CAEV sequences from peripheral blood mononuclear cells (PBMC), synovial fluid cells (SFC), and milk cells (MC) from the infected goats. This type of PCR assay especially provides a useful method to detect CAEV infection in goats (66-68). A two-step TaqMan quantitative (q) PCR, which is specific as well as sensitive for the detection of infection due to CAEV by the use of a set of primers (specific), and a TaqMan probe that targets a region which is highly conserved within the gene that encodes the capsid protein of the virus have been developed (33). In the total deoxyribonucleotide (DNA) extracts, the proviral DNA can be detected successfully by this assay. The TaqMan qPCR assay provides a fast as well as specific and sensitive means for detection of proviral DNA of the virus and thereby proves to be useful for detection in large scale for eradication programs as well as epidemiological studies.

PCR techniques have been standardizedin several laboratories for the detection of proviral DNA. Other molecular techniques such as cloning and sequencing are also used to provide knowledge on a country or region's specific strain of CAEV. Phylogenetic analyses of the proviral DNAs of CAEV throughout the world have given the suggestion that in certain areas CAEV causes natural infection not only in goats but also in sheep. In order to track the transmission of the disease in near future, phylogenetic analyses may be used(66,69,70). Molecular techniques such as cloning and sequencing are also used to provide knowledge on the prevalence of specific strain of CAEV in a country or a region which may have influence on serological assay as well as corresponding CAEV antigen (33, 71).

4.5. Ovine Progressive Pneumonia (Maedi-Visna)

Most of the sheep suffering from Ovine progressive pneumonia (OPP) do not show the clinical signs until the age of 2 years due to the long incubation period of the virus. General loss in body condition known as the “thin ewe syndrome” is the first sign of the disease. There may be loss of weight in spite of the normal appetite of the affected sheep (143, 144). Several serological tests like agar gel immunodiffusion (AGID), immunoprecipitation (IP), and competitive ELISA (cELISA) are used for the diagnosis of Ovine progressive pneumonia with the use of methionine-labelled antigen A (73). Real-time quantitative PCR (qPCR) which is specific for the transmembrane region of the envelope gene (tm) has been compared with competitive inhibition enzyme-linked immunosorbent assay (cELISA) using sheep sera. The qPCR assay indicates excellent agreement between the two tests. Both disrupted whole virus and recombinant viral proteins have been utilized in indirect ELISAs which have shown high sensitivity as well as specificity of detection [73]. Such experiments have proved that the proviral loads of Ovine progressive pneumonia virus (OPPV) qPCR can be confirmed by cloning as well as sequencing and can be used as diagnostic tool for OPPV infection as well as measurement of viral load in

sheep which are infected (74, 75). Single enzyme-based automated immunohistochemical (IHC) analysis has been developed to detect capsid antigen (CA) of OPPV that uses two anti-CAEV monoclonal antibodies, namely, 5A1 as well as 10A1 along with two enzyme-based IHC systems. The CA of OPPV has been detected in the intracellular regions of the synovial membrane of the carpus, in the cells that resemble alveolar macrophages as well as interstitial macrophages in the lung tissue, and so also in alveolar cells of the mammary gland (76). Comparison of a new real-time quantitative PCR (qPCR) which is specific for the envelope gene's transmembrane region has been done with a competitive ELISA (cELISA). Such comparative test has led to the conclusion that qPCR may be used as a supplemental tool for diagnosis and for measuring the load of the virus (71,145).

4.6. Enzootic Nasal Tumors and Ovine Pulmonary Adenomatosis (Jaagsiekte)

From the diagnostic point of view of enzootic nasal tumors and ovine pulmonary adenomatosis, it is important to note that the genome of the ovine pulmonary adenomatosis virus is 7,434 nucleotides long thereby exhibiting a genetic organization of type B as well as D oncoviruses. The enzootic nasal tumor virus is closely related to the Jaagsiekte retrovirus of sheep as well as to sheep endogenous retroviruses (145, 147). Diagnosis of enzootic nasal tumors is based on mainly clinical findings. Endoscopy reveals occlusion in the caudal part of one or both the nasal cavities. Radiography may also reveal the extent of the lesion. Provisional diagnosis can be made by the biopsy of the mass during the period of endoscopic examination [80]. RT-PCR for the diagnosis of Jaagsiekte is very important in order to formulate prevention as well as control strategies. The envelope (env) gene is mainly targeted for this purpose (81). For development of an assay based on serology, identification of three proteins has been done as candidate diagnostic antigens, namely, Jaagsiekte sheep retrovirus (JSRV) p26 (which is a group specific antigen), the transmembrane, and the open reading frame (ORF)-X proteins. Isolation of the genes coding for all the three proteins has been done followed by cloning as well as expression. Purification of the JSRV p26 has been done as a potential diagnostic antigen by both Western blot and ELISA. Investigation of three molecular assays has been done for their sensitivity as well as specificity: the long terminal repeat (LTR) group specific antigen (gag) PCR, LTR heminested PCR, and the PCR covering the V1 or V2 region. The use of AmpliTaq gold DNA polymerase increases the specificity of heminested PCR. The complete genome sequence of the ovine enzootic nasal tumor virus has been done which has shown its exclusive association with contagious intranasal tumors of sheep (79,82, 83).

4.7. Enzootic Pneumonia or Shipping Fever

Before discussing enzootic pneumonia in sheep, it has to be kept in mind that as far as the transmission of the disease from diseased to healthy animals is concerned, no direct evidence is available yet. As per suggestion, it has been noted that there may be precipitation of outbreaks due to abrupt environmental changes and it may also be associated with a sharp change in weather conditions (86, 87). Such infection in animals caused by a bacterial species related to genus *Pasteurella* is known as Pasteurellosis. After the taxonomic revision in 1999, the species is classified as *Mannheimia species*. *Pasteurella multocida (P. septica)*is carried in mouth and respiratory tract of several animals, notably cats. The organisms are small Gram-negative bacillus with bipolar staining. *P. multocida*, a common commensal, causes numerous pathological conditions in domestic animals, avian species, and human beings. Pasteurellosis is associated with a close animal contact and may be transmitted by animal bite (88,89). Severe clinical conditions occur when the organism is associated with other infectious agents, such as mycoplasma, chlamydia, and viruses (7,9). Environmental conditions and various stress factors such as transportation, housing deficiency, and bad weather also play a role to further aggravate the clinical conditions. Among the various diseases considered to be caused by *P. multocida*, alone or in association with other pathogens, most important is shipping fever in cattle and sheep, which may also be caused by *Mannheimia haemolytica*, in the absence of *P. multocida*. Fresh samples are the prerequisites for isolation of *Pasteurella multocida* and subsequently demonstration of the bipolar staining characteristic. A wide range of media that can be used for isolation of the organism are blood and chocolate agar and casein/sucrose/yeast (CSY) agar with supplementation of 5% blood. Other media include dextrose starch agar as well as trypticase soy agar. For demonstration of the characteristic staining feature, methylene blue or Leishman's stain is usually used. For serotyping, the tests include rapid slide agglutination test as well as indirect haemagglutination test (for capsular typing); for somatic typing an agglutination test; and agar gel immunodiffusion for both capsular and somatic typing. For the rapid identification of capsular type, counterimmunoelectrophoresis is an important diagnostic tool. Dot immunobinding assay, immunoblotting of outer membrane proteins of vaccine, and field isolates of *Pasteurella multocida* have been used for rapid diagnosis (90, 91). Comparative analysis of the outer membrane protein profiles of haemorrhagic septicaemia associated *P. multocida* by immunoblotting studies indicated that the major OMP of *P. multocida* (B: 2) is highly antigenic and 37 kDa OMP has potential for protective and immunodiagnostic studies(92).

In clinical samples as well as bacterial cultures, detection of organisms can be done by PCR. The pair of primers for this particular assay can amplify a 353 base pair (bp) fragment of the 16srRNA gene, which ultimately results in the amplification of DNA. Thus, this kind of PCR assay usually represents a valuable tool for diagnosing the disease early ultimately facilitating

better control of the disease. Similar strategies can be adopted for the identification and confirmation of enzootic pneumonia in sheep with advanced molecular methods(20, 35).

For epidemiological investigations, characterization of isolates can be done by DNA fingerprinting but availability of such diagnostic test is restricted to research laboratories (85, 93). Southern hybridization can lead to confirmation of the presence of the bacterial sequence, which is often suggestive of the virulence of the organism (94). Upon presumptive or definitive diagnosis, further differentiation of isolates can be achieved by genotypic fingerprinting methods. Restriction endonuclease analysis for characterization of serotypes of hemorrhagic septicaemia can be done with the enzyme HhaI. Discrimination of the isolates can be done by application of ribotyping as well as large DNA separation by means of pulsed-field gel electrophoresis. The rapidity as well as reproducibility of AFLP is high with higher index of discrimination. PCR fingerprinting is feasible in any laboratory, which has got the PCR capability. RAPD analysis as well as arbitrarily primed PCR (AP-PCR) is found to be useful for epidemiological investigation. For discriminating sheep as well as goat isolates, repetitive sequence PCR is also found to be useful. Repetitive extragenic palindromic REP-PCR as well as single prime PCR has been found to be useful for differentiating various serogroups of the bacteria (95,96).

4.8. Caseous Lymphadenitis

The disease is caused by *Corynebacterium pseudotuberculosis*. There are two basic forms of caseous lymphadenitis, that is, internal form and external form. Most of the affected animals manifest both forms of the disease depending on the multiple factors that are age, physiological conditions, environmental factors, and managemental practices (148). There is obvious nodule formation under the skin as well as enlargement of peripheral lymph nodes in the external form. The affected lymph nodes along with the subcutaneous tissues are enlarged with thick as well as cheesy pus which may rupture outward spontaneously or during the process of shearing or dipping. The internal form of caseous lymphadenitis (CLA) is manifested by vague signs such as weight loss, poor productivity, and decrease in fertility (3, 148,149). For the detection of the causative agent, *Corynebacterium pseudotuberculosis*, in sheep and goats, a double antibody sandwich ELISA has been developed, which has been further modified for improving the sensitivity. The main objective of developing this test is to detect the presence of antibodies against the bacterial exotoxin. It has been found that six proteins with varying molecular mass ranging from 29 to 68 kilo Dalton (kDa) react with sera from both goats and sheep acquiring infection experimentally or naturally. For classification of the sera with inconclusive results, immunoblot analysis has been found to be valuable (100, 101). Quantification of interferon

gamma (IFN-γ) is essential for accurate diagnosis of the disease for which an ovine IFN-γ ELISA has been developed. The sensitivity of the assay is slightly more for sheep than in goats while the specificity of the assay is higher for goats than for sheep. It can thus be concluded that IFN-γ is a potential marker in order to determine the status of CLA infection in small ruminants (102). For the diagnosis of CLA, another novel strategy is the employment of PCR for identification of the bacteria isolated from abscesses (103). The PCR has been found to be both sensitive and specific in addition to its rapidity of detecting *C. pseudotuberculosis* from sheep that are naturally infected (99).

4.9. Mycoplasmosis

As far as the antigenic variation is concerned, mycoplasmas have complex mechanisms enabling them to evade the immune system. They thereby cause several clinical symptoms which are having significant economic effect on production of small ruminants(107). There are many species in genus *Mycoplasma*associated with pneumonic and respiratory conditions in small ruminants, namely, *Mycoplasma agalactiae*,*Mycoplasma mycoides subspecies mycoides*, *Mycoplasma bovis*, *Mycoplasma capri*, *Mycoplasma capripneumoniae*, *Mycoplasma capricolum*, *Mycoplasma putrefaciens*, and many others (7,9,104,106-108). Mycoplasma infection associated syndromes range from septicemia (acute) along with death to chronicity of infection that results in reduced production (150). Pneumonia accompanied by mastitis, keratoconjunctivitis, abortions, and arthritis is commonly observed in mycoplasma syndrome(7,9,151). The conventional methods for diagnosis of mycoplasmosis include isolation of caprine and ovine mycoplasma in modified Hank's Balanced Salt Solution Liquid Media (MBHS-L), followed by biochemical characterization and staining (7, 9, 105). Initially, serological tests like growth inhibition, agar gel immunodiffusion, counter current electrophoresis, complement fixation, PAGE, and others were performed (110). However, cross-reactivity of closely related species could not be differentiated by these serological tests (7-9,104). Immunobinding assay with polyclonal sera was able to differentiate closely related species (111). It was followed by preparation of different antigens and purification with PAGE and SDS-PAGE in an attempt to identify potent specific immunogenic proteins of diagnostic values (112, 113). Moreover, detection of protective and cross-reactive proteins with SDS-PAGE and immunoblotting showed some glimpse of diagnostic value (9,114,115,151). These proteins provided base for selective and specific tests. Development of monoclonal antibodies based on such purified and specific immunogenic proteins led to development of very sensitive and specific sandwich ELISA based on monoclonal antibodies (116). Molecular detection of *Mycoplasma* species based on different set of primers was used to identify different species (26). For the development of monoclonal

antibody based serological as well as ELISA-PCR, identification of species specific non-cross-reactive immunogenic proteins is mandatory, and for that proteins separated in SDS-PAGE were subjected to western blotting with homo- and heterologous sera against *Mycoplasma agalactiae* and *Mycoplasma bovis* (9,114, 115, 151). These species specific immunogenic proteins can form the basis for development of many advanced diagnostic procedures for the detection of mycoplasma and its species confirmation.

Nowadays, for the molecular diagnosis of several clusters as well as groups, species specific primers along with restriction enzymes are used for confirmation of the agent by PCR as well as PCR-RFLP (107). Still the combination of conventional and recently developed molecular methods is recommended for the identification and confirmation of contagious caprine pleuropneumonia (CCPP) in field outbreak (117). For this purpose, growth inhibition test has been employed for identification of the agent followed by PCR. These two tests in particular detect two species of *Mycoplasma*, namely, *Mycoplasma capricolum* and*Mycoplasma putrefaciens* from nasal swab and lung cultures (119). A multiplex real-time PCR has been developed for differentiation of the various *Mycoplasma* species of sheep and goat including *Mycoplasma agalactiae*. This assay particularly targets the two specific housekeeping genes, namely, polC and fusA considering which specific diagnostic primers and probes are to be developed (105). It is however important to note that the assay requires further assessment of clinical specimens but for diagnosis on large scale basis the assay is very promising (119). Primers specific to *Mycoplasma conjunctivae* (that causes pink eye in sheep and goat) have been used for amplification of a 750-base-pair fragment of the genome through PCR, which has been subsequently confirmed by agarose gel electrophoresis (107, 109).

4.10. Nasal Myiasis

Both double immunodiffusion (DD) and indirect haemagglutination (IH) tests are used for detection of the somatic crude antigen first (L1) as well as second (L2) and third (L3) in star of the larva of the parasite*Oestrus ovis* (122). For postmortem examination, sagittal sectioning of the head of the sheep suspected of suffering from nasal myiasis is carried out for detecting the presence of maggots or larvae (152). It has been observed that there is no development of cross-immune reaction in sheep, which are naturally parasitized with all the three larval stages (as detected by DD test) and with L2 larvae (as detected by IH test) (124). It is important to note that rhinoscopy examination can confirm the diagnosis and is equally important in treating the patient by removing the maggots with forceps (123). For detection of seropositivity, ELISA is employed using a crude L2 larva as antigen (121). Development of a direct ELISA by the use of a crude somatic antigen was developed from the first stage larva (L1). Validation of such system has

been done with sera from both endemic and nonendemic areas (125). The sensitivity as well as the specificity of the assay has been found to be high by the use of a cut-off point. PCR as well as automated sequencing technologies have been developed for molecular diagnosis of the disease (128). PCR-RFLP has been used widely for identifying taxa of the parasite which are closely related and have forensic relevance (120). It is also important to note that a better understanding of several target genes like mitochondrial DNA (mt DNA) as well as ribosomal DNA (rDNA) is pertinent for understanding the evolution of the parasite and so also for characterization of the proteins of the parasites (120, 123).

4.11. Verminous Pneumonia

In goats *Muellerius capillaris* is the most common lung worm. There is diffused pneumonia in affected goats without the presence of any nodular lesion. The parasite predisposes animals to secondary infections thereby compromising with the health in general (129). A rapid as well as inexpensive method for assessment of herd exposure to lung worm in cattle is the bulk milk ELISA. It is a useful tool for the veterinary practitioners as a herd health monitoring programme component or in the perspective of investigation of herd health (126). Over the past 15 years, studies have been conducted to prove that sequences of the internal transcribed spacers of ribosomal DNA provide useful genetic markers. This makes the basis for the molecular diagnosis of parasitic pneumonia in sheep and goat using PCR (130). DNA probes as well as assays based on PCR are used for identification and detection of *Dictyocaulus* as well as *Protostrongylus*. The sensitivity of most of the PCR-based assays is more than DNA probe assays. Multiple steps are required for the development of assays based on PCR, which follows the selection of oligonucleotide primers at the initial stage along with reporter probe. It has been found that usually PCR detects the parasitic DNA but certainly advances have been made in preparing samples. For this purpose, it is required to extract the DNA while removing the PCR inhibitors. This helps in achieving greater sensitivity (128).

Go to:

5. Other Unusual Complications of Respiratory Tracts

The respiratory diseases of small ruminants are generally fatal to lambs and kids. The lamb and kid pneumonia are mostly regarded as a complex of disease. It involves interaction of host related factors (immunological and physiological) and etiological agents, namely, virus, bacteria, mycoplasma, and environmental factors (4,7,108). Many times, immunosuppression, malnutrition, and adverse climatic conditions lead to infection due to unusual infectious agents. There are reports on *Streptococcus pneumoniae*, commensal bacteria of the nasopharynx of animals associated with a majority of cases of morbidity and mortality in young lambs due to

pneumonia (7,13,153). Similarly, many other unusual pathogens *Haemophilus ovis* (154), *Streptococcus* spp., *Pasteurella* spp. (5,6), *M. bovis* in sheep (7,12), and goat (155), *Mycoplasma arginini* (12), and *Haemophilus somnus (156)* may cause pneumonia. Many time mixed infections are observed. Thus, isolation and identification of such samples are always tedious to perform (7,9). The use of monoclonal antibodies based serological tests has simplified the process of early and specific diagnosis of many of these pathogens (24). Simultaneously, development of molecular techniques like PCR particularly multiplex PCR is very useful for the identification and differentiation of etiological agents from such complex conditions (21).

Materials and methods

Samples collection and bacterial culture

This study was conducted in Samawah city / Al Muthanna Government 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.2).

Figure. 2: Shows the examination of the animal before slaghtering

Before slaughtering all animals were examined for any signs of respiratory diseases. One hundred four nasal swabs were collected from nasopharyngeal area of sheep and goats that showed nasal discharge (Figure.3).

Figure.3: Shows nasal swab collection from infected sheep.

The nasal samples were collected by inserting sterile cotton-tipped applicator sticks or swab into the nasal passage after proper cleaning and disinfection of the external wares. Each nasal swab was carefully cut and put into a labeled bottle containing 2 mL brain heart infusion broth. The swabs were transported in a cool box to the laboratory for bacterial culture. Moreover, lung and tracheal samples were collected also from 5 dead animals in 2 herds of sheep that suffered from

high morbidity and mortality rate due to respiratory infection. After, the samples were arrived to the clinical pathology laboratory/ college of veterinary medicine/ Al Muthanna University. Then, the samples that cultured in brain heart infusion broth (BHIB\Himedia, India) were kept at 37˚C for 24 hr, and inoculated on blood agar (B.A\Himedia, India) with 5% sheep blood and MacConkey agar (M.A\Himedia, India), then incubated under anaerobic condition at 37˚C for 24 h with 5% Co_2. 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 (18, 19) (Figure. 4, 5 , 6).

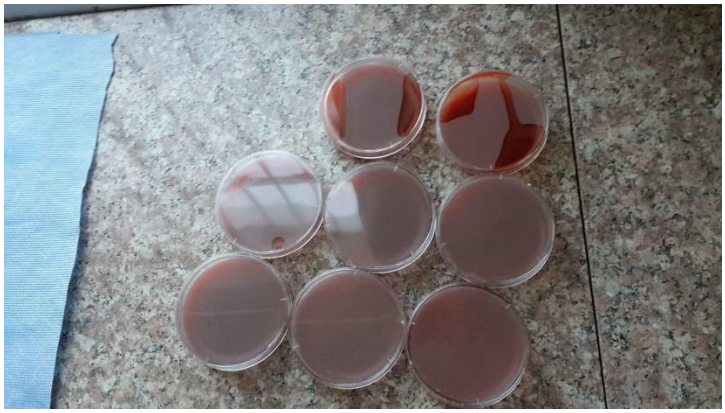

Figure.4: Shows blood agar use for culture of samples

Figure. 5: Shows the TSI use for identification of bacteria.

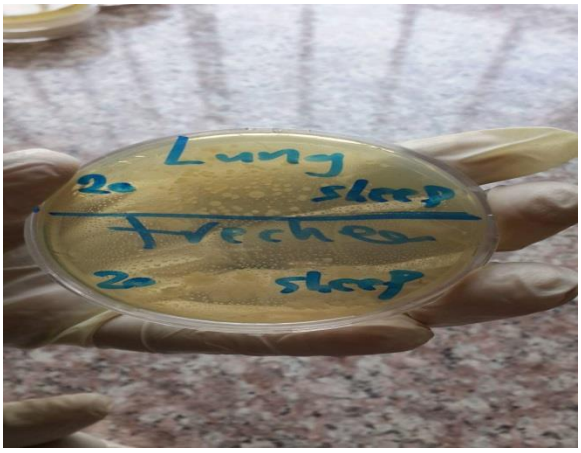

Figure. 6: Shows the culture of trachea and lung on nutrient agar.

After taking note of cultural growth characteristics, positive cultures were subjected to Gram's staining properties and cellular morphology observed with alight microscope (x100). Mixed colonies and Gram negative bacteria were subcultured on both blood and McConkey agars and further incubated aerobically for 24 h. Pure culture of single colony type from both blood and McConkey agars were transferred onto nutrient agar slants for a series of biochemical tests including catalase, oxidase and fermentative/oxidative tests for final identification following standard procedures (21).

Histopathological examination

The animals with respiratory signs were examined grossly and lung lesions were identified (Figure. 7). All samples with gross feature of and revealed different pathological lesions were taken from infected lung of sheep and goats. The lesions were examined macroscopically and histopathologically. A gross inspection of lesions with respect to the shape, size, color and consistency of the lesions were recorded. 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 according to (8).

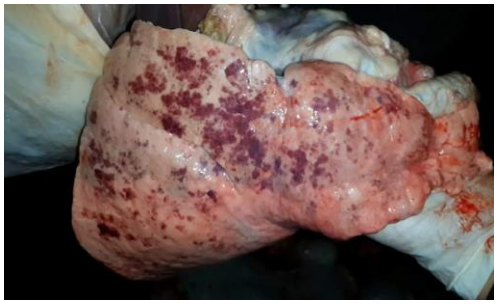

Figure. 7: shows the macroscopical examination of lungs lesions

Molecular Identification of *M. haemolytica*

DNA Extraction

Bacterial cells (up to 1×10^9) were cultured, in BHI broth, and overnight incubated, then were transferred to1.5 ml micro centrifuge tube then centrifuged at 16,000 g for 1 min, DNA was extracted using Presto Mini g DNA bacteria Kit according to manufacturer's instructions (Geneaid, KOBA). The extracted DNA was stored at −20˚C until use. The DNA concentration was measure by NANODROP-2000 spectrophotometer (Thermo Scientific Inc., USA).

Primers

Oligonucleotide primers for *M. haemolytica* were obtained from IDT (Integrated DNA Technologies/USA). The primer sequence of M. haemolytica 16SrDNA gene was (F-GCTAACTCCGTGCCAGCAG, R-CGTGGACTACCAG GGTATCTAATC) with size 304 bp (16) and the sequence of 12s rRNA gene was (F-TAACCCTTGTMCCTTTTGSATRRK, R-AGACTAACTTTTAAAGATACA GTGGG) with size 270 bp (17) .

PCR Amplification Analysis

PCR amplification was performed on a final volume of 20 µl containing 10 Intron-Master Mix (KOBA) which contains (Taq plymerase, PCR buffer, Gel loading buffer and dNTPs), 2 µl (100 ng of DNA template) and 2 µl of 10 pmol for each primer. The amplification of gene was carried out 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 and 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. 2). The diseased animals was 104 out of 270 in addition to the five dead animals. The percentages of the respiratory diseases were 38.51 % including 29/ 62 (46.77 %) and 33/62 (53. 22%) for male and female respectively in sheep and totally 62/107 (57. 94%). The percentages of respiratory diseases in goat were 23/42 (54.76%) and 19 /42 (45.23%), and totally 42/ 163 (25.76%).

Table.2: 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

The aerobic isolated bacteria associated with respiratory in small ruminants were *Mannheimia haemolytica, Escherichia coli, Pasteurella multocida, Klebsiella pneumoniae and Staphylococcus aureus and Streptococcus pyogenes*.

Identification of *M. haemolytica* was done by study colonial morphology on B.A and M.A and showed minor differences between field isolates and standard strain. Lung isolates appeared on B.A as small, gray and rough colonies. While nasal isolates appeared as large, gray and mucoid (smooth) colonies (Figure.8). Lung and nasal isolates showed beta haemolysis on the B.A after 24 h. Lung isolates appeared as Pink pinpoint colonies on M.A, while nasal isolate appeared as mucoid pink colonies. While, standard strain of *M. haemolytica* appeared as small, gray and rough colonies on B.A haemolysis appeared after 48 h under neath the colony, and as dark pink pinpoint colonies on M.A. 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.

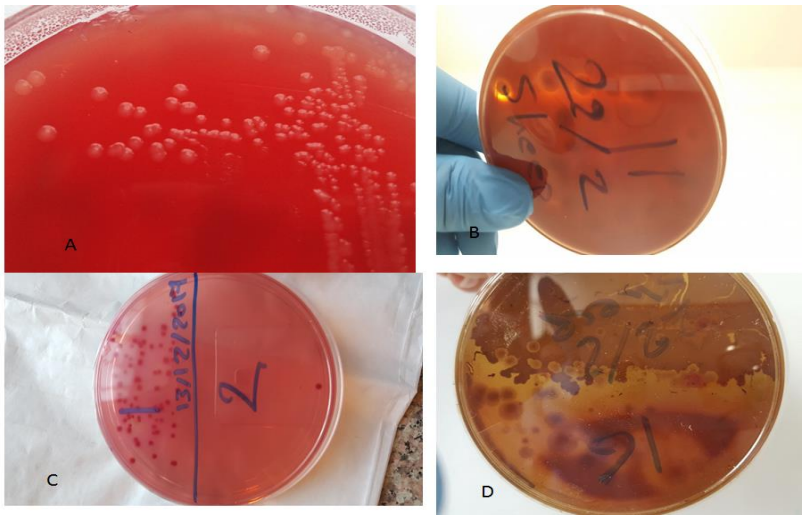

Figure.8: Shows the growth of : A. *Pasteurella multocida*, *B & D Mannheimia. haemolytica* , *C*. Pink pinpoint colonies of *Mannheimia. haemolytica* on M.A

The gross lesions observed were majorly, suppurative pneumonia, exudative pneumonia, congestion (Figure 9), and various stages of pneumonia.

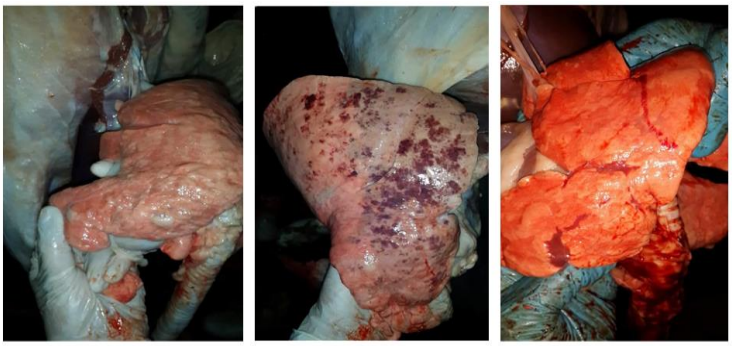

Figure. 9: shows various gross lesions on lung, from left emphysema and nodule, congestions, red hepatization

Histologically, the following types of pneumonia was seen : suppurative bronchopneumonia necrotic bronchopneumonia, fibrinous bronchopneumonia, bronchointerstitial pneumonia, and pyogranulomatous pneumonia. The lungs diagnosed with bronchopneumonia were characterised by neutrophilic exudates were present in the alveolar spaces and lumens of the bronchioles and bronchi, and in some occasions a mixture of various amounts of cell debris, neutrophils and macrophages were observed in these areas and there are also distended interlobular space, infiltrated with inflammatory cells, distended alveoli 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. 10).

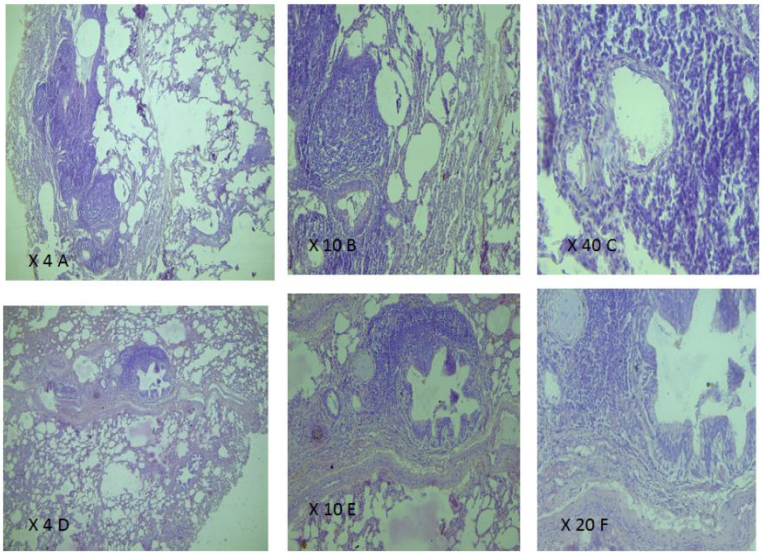

Figure.10: pyogranulomatous pneumonia (A, B & C); bronchointerstitial pneumonia (D, E, F).

Identification *M. haemolytica* was Confirm for all strains of by PCR analysis. The test was performed, all isolates were tested to present 16 s rDNA and 12 s rRNA genes. Hereafter, all strains were positive for all isolated bacteria and 12 s primers, they showed a specific 304 bp and 270 band respectively on agarose gel, no amplification was observed in control negative (11).

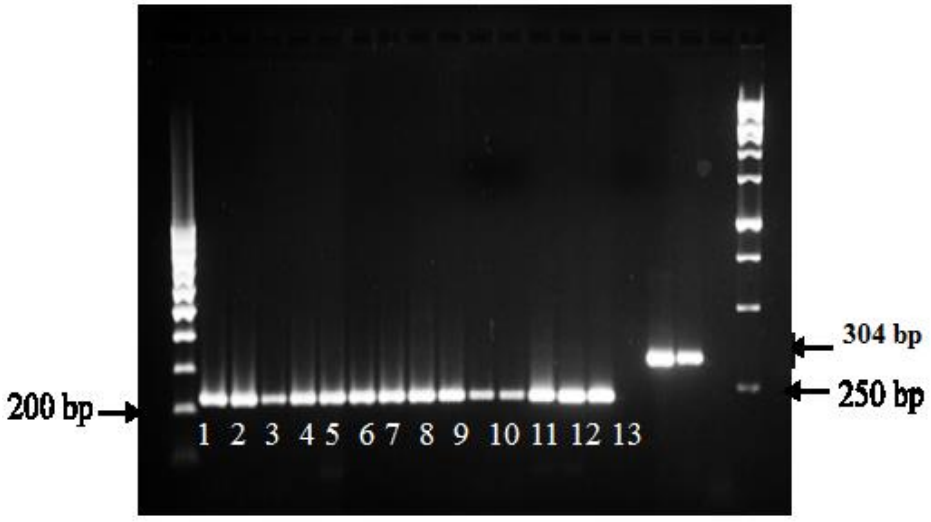

Figure. 11. Agarose gel electrophoresis (1%) of amplified 16 srRNA gene (304 bp) of *M haemolytica* , stained with ethidum bromide, Lane M: 100 bp ladder (1500 bp) positive *M. haemolytica* isolates and the standard strain.

Discussion

RESPIRATORY disease is commonly encountered in sheep flocks, affecting groups or individuals. It often involves a combination of infectious causes as well as predisposing management factors, potentially leading to significant losses. However, cases of respiratory disease represent only 5·6 per cent of sheep submissions received by the Veterinary Laboratories Agency – a figure that is likely to be lower than the actual prevalence. In many instances, a presumptive diagnosis will be made following clinical and on-farm postmortem examinations.

This study showed that a variety of bacterial colonized the nasal passage ways of diseased sheep and goats. Several authors reported similar bacteria from pneumonic lungs in goats and sheep (1, 13). The isolation of S. aureus from the nasal passage is consistent with other findings obtained from caprine (25) and ovine (7) lungs. Robbins et al. (23) report that *S. aureus* resides in the upper respiratory tract and is involved in disease processes only when stress conditions prevail. The detection of *E. coli* in nasal samples of goats is also consistent with findings by other authors (20). *E. coli*, which is known to be usually harmless in its normal habitat, can cause pulmonary and urogenital tract infection (20). This may also be associated with possible fecal contamination due to the sniffing nature of goats, especially those on heat and during courting before mating.

The constant isolation of *M. haemolytica* from the lungs of various animal species either healthy or having different respiratory syndromes may indicate their possible role in infectious pneumopathies (16). *M. haemolytica* was isolated in the nasopharynx and tonsils of apparently healthy animals, where, interestingly, serotype A2 is most commonly isolated from both sheep and cattle (24). The

organism can be isolated from lambs soon after birth (5) and this carriage has been shown by Pass and Thompson (18) to fluctuate over time. The presence of the organism in the nasopharynx of sheep has been shown to coincide with the occurrence of infections. In the nasal passages of calves the bacterial flora has been shown to fluctuate in both species and numbers and, although *M. haemolytica* can dominate the flora, it can also be absent for weeks at a time (15). There has also been failure to culture consistently *M. haemolytica* in swabs taken daily from known colonized animals (18). The mechanisms that *M. haemolytica* possesses to survive in the upper respiratory tract are unknown (24). Stress factors with or without viral infection have been reported to suppress the mucociliary clearance mechanism which allows the proliferation of bacterial commensals in the respiratory tract (8). They also cause an abrupt shift from commensal to pathogen especially in *M. haemolytica* where serotype 2 shifts to serotype 1, which is known to be pathogenic to animals (12). This shift has made *M. haemolytica* to assume greater prominence in caprine pneumonia (16). Considering the stress of weather, disease and poor management conditions to which the animals are constantly subjected, the pathogenic role of several bacterial species, and especially *M. haemolytica*, that inhabit the upper respiratory tract of apparently normal sheep and goat. Apart from the possible pathogenic role of the normal nasal bacterial flora, drug resistance of some the pathogenic bacteria has become a rampant, proven, serious problem to both animal and human health care providers. Aghomo and Ojo (2) reported a high level of resistance of *M. haemolytica* to streptomycin while the organism was found to be susceptible to ampicillin, oxytetracycline, and chloramphenicol.

Previous investigations on the prevalence of *M. haemolytica* have shown a considerable variation. Ranges between 8.9% and 96.2% of healthy sheep that carry these organisms in the nasal cavity have been reported (AL-Tarazi and Dagnall 1997; Biperstein *et al.*, 1966, 1970).

The variation is likely to be caused by several factors including different isolation techniques, misidentification, and seasonal variation. In the present study, swabbing of the tonsils and nasal cavity of slaughtered sheep and goat showed that *M. haemolytica* was isolated from nasopharyngeal swabs and this result is in agreement with (Gilmour *et al.*, 1974).
Furthermore, it has been found that the prevalence of *M. haemolytica* in temperate climates varies seasonally with a higher prevalence in spring and early summer (Gilmour and Gilmour, 1989). Frequencies of *Mannheimia* strain isolates are very variable and can sometimes be high and differ depending on the source of isolation: United States, 15.8% in sheep nasal exudates (Frank, 1982), United Kingdom, recorded a prevalence of 52% for *Mannheimia* and 42% for *P. trehalosi* in sheep and goats respiratory system and Turkey, 8.3% in ovine lungs (Kirkan and Kaya, 2005). The prevalence of *M. haemolytica* was lower than that found in ovine nasal exudates recorded by Blanco *et al.*, 1995 (25%); Pijoan *et al.*, (1999) (35%).It is evident that *M. haemolytica* isolates predominate over *P. trehalosi* in both species. Bali *et al.*, 1993 reported that, although *M. haemolytica* was the most frequent serotype isolated from sheep in Northern Ireland, *P. trehalosi* outnumbered *M. haemolytica*. They constituted 45.4% vs. 38.8% for *M. haemolytica*, while untypable strains represented 15.8%. Odendaal and Henton, 1995 reported that, although most serotypes were present in sheep and goats in South Africa, *M. haemolytica* serotypes predominated over the *P. trehalosi* strains (49.8% vs. 16.4%). The majority of serotypes were associated
with pneumonia, followed by gangrenous mastitis"blue udder" and septicemia. β-haemolytic *Mannheimia* species were isolated from 24% to 64% of the sheep in four flocks of sheep in Norway, a total of 26 haemolytic M. ruminalis-like strains were isolated among which, a considerable genetic diversity was found (Poulsen et al., 2006). *M. haemolytica* causes sporadic cases and small outbreaks of acute pneumonia and pleuritis in goat kids (Jubb et al., 1993). However, little is known about the epidemiology of pasteurellosis in goats. According to Gilmour and Gilmour, 1989 M. haemolytica is normally associated with pneumonia in cattle and sheep, septicaemia in lambs and mastitis in ewes. These observations have subsequently been supported by Angen *et al.*, 2002; Garcia et al., 2009.
However, the present investigation clearly demonstrated that these organisms also can be obtained from the upper respiratory tract of apparently healthy sheep.

The major types of pneumonia namely bronchopneumonia and interstitial pneumonia were observed during the histopathological examination. The types of pneumonia observed were the most common types of pneumonia encountered in most studies. Tijjani et al. (2012) reported bacterial flora and pathologic lesions of caprine pneumonic lungs in Maiduguri, Nigeria, Yesuf et al. (2012) had equally reported histopathological changes and bacterial flora associated with pneumonic lungs of small ruminants slaughtered at Gondar, Ethiopia, and Ashraf et al. (1986) had reported incidence and pathology of pneumonias 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. For decades, microbiologists have sought improved pathogen identification through the use of phenotypic methods, but they still rely on phenotypic identification.
Phenotypic tests were used in current study for identification of *M. haemolytica* . All characterization results of phenotypic tests for *M. haemolytica* (field isolates and standard strain)

are in agreement with [18] who reported that the *M. haemolytica* appeared as a large or small, gray and rough or smooth colonies appeared on blood agar with haemolysis which appeared after 24 - 48 hr around or under neath the colonies, while it appeared as pink to red pinpoint colonies on MacConkey agar, except the nasal mucus isolate appeared as mucoid pink colonies on MacConkey agar, this result is closely agreement with (5) Also, the characterization of *M. haemolytica* isolates is in agreement with the findings reported by (33) that the isolate belong to *M. haemolytica* did not produce indole and grew on MacConkey agar. The *M. haemolytica* isolates were positively identified by conventional biochemical's. The results were agreement with (39) that proved the lower reliability of this system for the identification of *M. haemolytica* . All the isolates and standard strain of *M. haemolytica* showed positive results for 16s rRNA gene and corresponds approximately size to 304bp, this result is in agreement with finding of [16] were used the same primer which successively amplified to 304 bp and to sequencing as *M. haemolytica*. In addition, when 12s rRNA gene was used, all isolates also showed positive results and corresponds to anticipate size 270 bp. This result was in agreement with [17] who detected *M. haemolytica* directly from lung tissues and from bacterial culture by used 12s rRNA gene.

Conclusions

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 microorgaisms that might be the cause of respiratory diseases in this study, moreover, *M. haemolytica* showed a positive results with PCR. The author, recommoned to consider PCR as a valuable tool for rapid detection of *M. haemolytica* in clinical samples from sheep and goats. In addition, 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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