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Isolation and molecular diagnosis of the main bacterial species causing Pneumonia in small ruminants in the Duhok Abattoir-Kurdistan region of Iraq

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ABSTRACT

Diseases of the respiratory system are common in all species of domestic animals, and there are many different factors acting together to cause the appearance of the disease which results in economical loss. This study was aimed to describe the prevalence of bacterial agents, isolation, identification, and molecular diagnosis in Duhok Province This study was conducted on 4061 slaughtered animals which were divided into sheep (3667) and (394) goats at Duhok abattoir, from September 2020 to September 2021. Two hundred fifty-three affected lungs were collected from slaughters of sheep and goats. Different media and biochemical tests were used for the isolation and identification of different types of bacterial species from affected lung samples. The results of bacterial isolation detected seven different species of gram-positive and negative bacteria. The result found that highest percentage rate was recorded with *Staphylococcus aureus* while lowest rate was with *Pseudomonous* spp they were identified by colony morphology and the traditional biochemical examination. In conclusion, the current study found that there were different species of bacteria that causes pneumonia in small ruminant at the Duhok abattoir.

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Introduction

Abattoir or slaughterhouses surveillance has been a critical role in the control and eradication of infectious disease programs worldwide. Besides that, the slaughterhouse scrutinizing is an extremely beneficial mechanism to supervise the incidence rates of disease to emphasize and validate the diagnosis of suspected diseases (Al-Qudah et al. 2008). Small ruminants, especially sheep and goats coordinate significantly with the economy of farmers in Iraq. These small ruminants are valuable resources because of their potential to replicate and grow rapidly and their significant contribution to meat, milk, and wool production. Among these diseases inflammatory and non-inflammatory conditions affect the respiratory system. The respiratory tract system is a major organ that plays a crucial role in respiration called the ventilation system which is a limited gas exchanged via the respiratory construction and outside atmosphere (Yousif et al. 2019). The lung, the main organ of the respiratory system, is susceptible to many infectious and non-infectious agents causing different pathological conditions in farm animals (Alam et al. 2001; Ferdausi et al. 2008). Pneumonia can be caused by direct infection with bacteria by arriving haematogenously or by inhalation. In much pulmonary inflammation, a sudden alteration in the micro normal of the nasal or upper respiratory system with a dramatic augment in one or more species is the trigger for a pulmonary infection (Habasha et al. 2016).

Within the Asia region, mainly in countries like Iraq, at the beginning of the winter season, or just before the lush or extreme producing season, outbreaks of acute respiratory infections of pasteurellosis disease have been noted to occur in sheep and goats (Rasha et al. 2014; Chakrabortyet et al.2014). Some study conducted to

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analyze pneumonia caused by bacterial pathogens (Sen 2018). As well as some bacteria such as Pasteurella spp. and Staphylococcus spp. are normally resident in the respiratory tract of healthy sheep and goats. However, they occasionally cause pneumonia during stress conditions or are attributable to other pathogens. Furthermore, Klebsiella spp. causes pneumonia, while it produces virulence factors such as smooth lipopolysaccharide, pili for adhesive to host cells, and capsules (K antigen) that are antiphagocytic assistance the bacterium in its struggle with the host for iron uptake (Ghanem et al. 2015; Izquierdo et al. 2003).

Domestic livestock animals, especially sheep and goats have been considered the majority principal slaughtered animals for human consumption in Iraq and by reason of differentiation of the geographic site, nutrition and climate are detecting factors on the type of microorganism causing pneumonia. Moreover, breeding systems, stress factors, environmental changes, unhygienic conditions, unexpected changes, and a low level of herd health status known as predisposing factors to bacteria, parasites, and viruses' infection which is recorded as the one common causative agents of pulmonary lesions and death of infected animals (Mahamud et al. 2017; Tiwari et al. 2021). Occasionally, Pneumonia termed goat "shipping fever" is one of the most prevalent problems faced today correlated with valuable treatments. Therefore, initial interpretation and convenient treatment are necessary. The current study aimed to describe the prevalence of bacterial agents, isolation, identification, and molecular diagnosis of bacterial causative agents from the lung lesions of sheep and goats at the abattoir in Duhok Province.

Materials & Methods

Study area

The present study was conducted from September 2020 to September 2021 in Kurdistan Region of Iraq at Duhok abattoir which is located in Seumel regain around 45 kilometers from Duhok city of Iraq. It is one of the major modernized abattoirs which export fresh meat to all of Duhok province. Most of the sheep and goats slaughtered in the abattoir and included in this study originated predominantly from different areas near Duhok city as well as imported from outside

Collection of samples and processing

The samples were collected from Duhok abattoir affected lungs were collected from native goats and sheep immediately after slaughter especially those had macroscopical lesions. Without details of sex breed or husbandry conditions of goats and sheep were obtainable. A total of 253 affected lungs were collected for the isolation of bacterial agents that cause pneumonia. All the specimens were transferred to the Histopathology, and Bacteriology laboratory, Duhok Research Center, College of Veterinary Medicine.

Isolation and identification of bacterial species

After collection of 253 affected the lungs of sheep and goats, the outer layer of pneumonic lungs was seared with a heated spatula before cutting the inside layer of the lung. The swabs were aseptically collected from the inner core of the lungs with a cotton pad and immediately placed into a Bijou bottle containing 10 ml of brain heart infusion broth and incubated at 37°C for 24 hrs. For sub-culturing, suspected bacteria were inoculated separately into various culture media under aseptic condition and afterwards incubated aerobically at 37°C for 24-48 hours. In this study different culture media including blood agar with the addition of 5% of sheep blood, MacConkey agar, and mannitol salt agar were used. Pure cultures for each strain were obtained. The identification of the microorganisms was performed by the biochemical tests as described by (Alam et al. 2001; Ferdausi et al. 2008; Tonu et al. 2011) according staining characteristics, the bacteria were classified into Gram-positive and Gram-negative

Isolation and identification of Pasteurellaceae family

For isolation and identification of Bacteria belonging to the Pasteurellaceae family, chromogenic agar (CHROMagar™ / France) was used and it is first chromogenic medium specific to detect Pasteurellaceae. The isolation was performed according to the manufacture instructions.

Biochemical test for isolated bacteria

To identify isolated bacteria, cultural, morphological, and biochemical characteristics were determined. Biochemical tests, such as cytochrome oxidase, catalase test, indole, urease production, hydrogen sulfide production (TSI), oxidation, and fermentation under aerobic conditions were used. The pure colonies were maintained in stocks containing brain heart infusion broth with glycerol 50% for a long period of time and for further investigations (Quinn et al. 1998).

Molecular identification

Extraction of DNA

The pure colonies of suspected bacteria from the selective media plate were transferred into Eppendorf tube (1.5ml) containing 500µL sterile deionized distilled water. The mixture was vortexed for a minimum of 15-30 seconds and directly heated at 100ºC for 15 min. Then, the suspensions were immediately cooled for 5 minutes. The mixture was then centrifuged at 15.000 rpm for 10 min (Arnada et al. 2004; Abdulrahman 2020; Abdulrahman 2021). The supernatant which contains the genomic DNA was transferred to sterile PCR tubes for PCR amplification. The purity of DNA and its concentration was checked by using a NanoDrop-Spectrophotometer. The PCR products were stored at -20 ˚C.

Conventional PCR for confirmation of isolated bacterial species

The presumptive phenotypic identification of isolated bacteria from pneumonic lungs of small ruminants based on standard biochemical tests was moreover confirmed by subjecting to PCR technique for amplification of different genes using species-specific primer Table 1 (supplementary file).

PCR amplification analysis and agarose electrophoresis

PCR was performed in a Thermocycler (Leica, Germany) in a total reaction volume of 25 µl. Each reaction consisted of12.5 µl of Crystal Hot start master mix (Jena Bioscience, Germany) Taq polymerase, nucleotides ATP, CTP, dGTP, dTTP), $KCl_2(NH4)_2SO_4$, $MgCl_2$ density reagent, enhancing and stabilizing additive), 1.5 µl of each of reverse and forward primer (10 pmol) and 4µl of sample DNA(30-100ng/ µl), and the remaining was completed with sterile deionized distilled water. Amplification conditions for each suspected bacterial species were performed according to Table 2 (supplementary file). The amplified PCR products were analyzed using1.5% agarose gel in a 1X TAE buffer and it was run at 100 V for 1 hr. Gels were photographed under UV illumination (Leica, Germany) after staining with 5µl/100mL safe dyes.

Results

Prevalence rate of pneumonia among sheep and goats

Among 4061 of the total examined animal's including 3667 sheep and 394goats, the present study showed that percentage of affected animals with pneumonia in sheep and goats was 86.17% and 13.83%, respectively. The results also found that the highest occurrence of pneumonia was recorded in sheep compared to goat as shown in Table 3 (supplementary file).

Prevalence of bacterial species isolated from infected animals

According to the traditional bacteriology methods, different bacterial species were identified from pneumonic cases in sheep and goats as shows in Table 4 (supplementary file). The isolated species from sheep and goat, respectively were as follow: *Pasturella multocida* (6% & 4%), *Mannheimia haemolytica* (2,4% & 2.6%), Escherichia coli (22% & 15%), *Staphylococcus aureus* (34% & 40%), *Klebsiella pneumonia* (4.8% & 4%), *Pseudomonous spp*. (0.8% & 2.6), *Proteus spp*. (2.8% & 4%) and mixed bacteria *Staphylococcus aureus* and *Escherichia coli* (16% & 13.33%) respectively Table 4 (supplementary file).

Biochemical tests

The biochemical investigation results are shown in Table 5 (supplementary file).

Cultural characteristics of isolated bacteria

The cultural characteristics of all isolated bacteria were varied as shown in Table 6 (supplementary file).

Molecular identification and confirmation of bacterial species

The suspected isolates by standard bacteriology methods were confirmed by using PCR. The results confirmed the identification of each suspected bacterial species from cases of pneumonia in sheep and goats as shown in Figures 1-4. During the preliminary study, the amplification of the reference and field was very specific to their species. The PCR was employed for molecular detection of the obtained bacterial species using standard and unique primer sets as shown in Table 1 (supplementary file). The PCR technique successfully amplified the target genes in all bacterial species that were isolated from the pneumonic lung. Furthermore, species specific genes including sodC, KMT1, uidA, gyrA, nuc, O-antigen acetylase gene and zapA were amplified successfully. The amplified products were of 230bp, 457bp, 147bp, 441bp, 270bp, 232bp, and 540bp, respectively. sodC, KMT1, uidA, gyrA, nuc, O-antigen acetylase gene and zapA were targeting *M. haemolytica, P. multocida, E. coli, Klebsiella pneumonia, Staphylococcus aureus, Pseudomonas aeruginosa and Proteus mirbilis*, respectively

Discussion

Respiratory systems diseases are common in different species of domestic animals, specifically herbivores. Because their major economic effect on the sheep and goat industries, through consequences such as death, retarded growth and decrease weight-gains in recovered animals, in addition to slaughterhouse wastage, medication, and labor outlay, the pneumonia caused by bacteria has been broadly studied experimentally and in the field (Azizi et al.2013). Estimating the prevalence and outbreak of lung lesions and their influence on the growth of lambs and kids showed that severe lung lesions perhaps lead to a greatly reduced growth performance of the animals. The lack of data and studies that deal with pneumonia in small ruminants in Iraq and Kurdistan Region may be one of the reasons for the spreading of respiratory diseases in the field of small ruminants. (Daniel et al.2006). The small ruminants, especially sheep and goats play an essential economic role as they are raised mostly for lamb production, subsequently wool and milk for a large section of the population, particularly in village and desert areas. Therefore, they can support the survival of millions of people in numerous countries all over the world including Egypt and Iraq (Hatem et al. 2003; Ali et al. 2009).

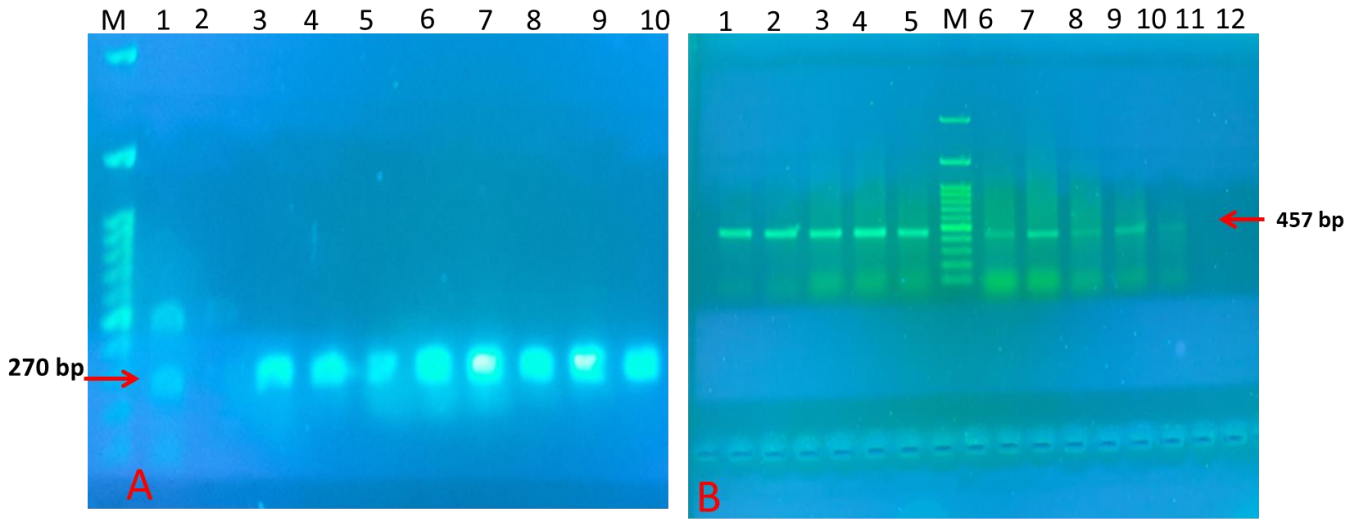

Fig 1. Panel (A) PCR amplification of nuc (270) bp of *S. aureus*, Lanes 1 and 2 represented negative samples, lanes 3-10 represented positive samples, Panel (B) PCR amplification of KMT1(457) bp457 of *P. multocida*, lanes 11 and 12 represented negative samples and lanes 1-10 represented positive samples. Molecular weight marker: M=pBr322/Msp1.5% agarose gels and stained with Safe dye.

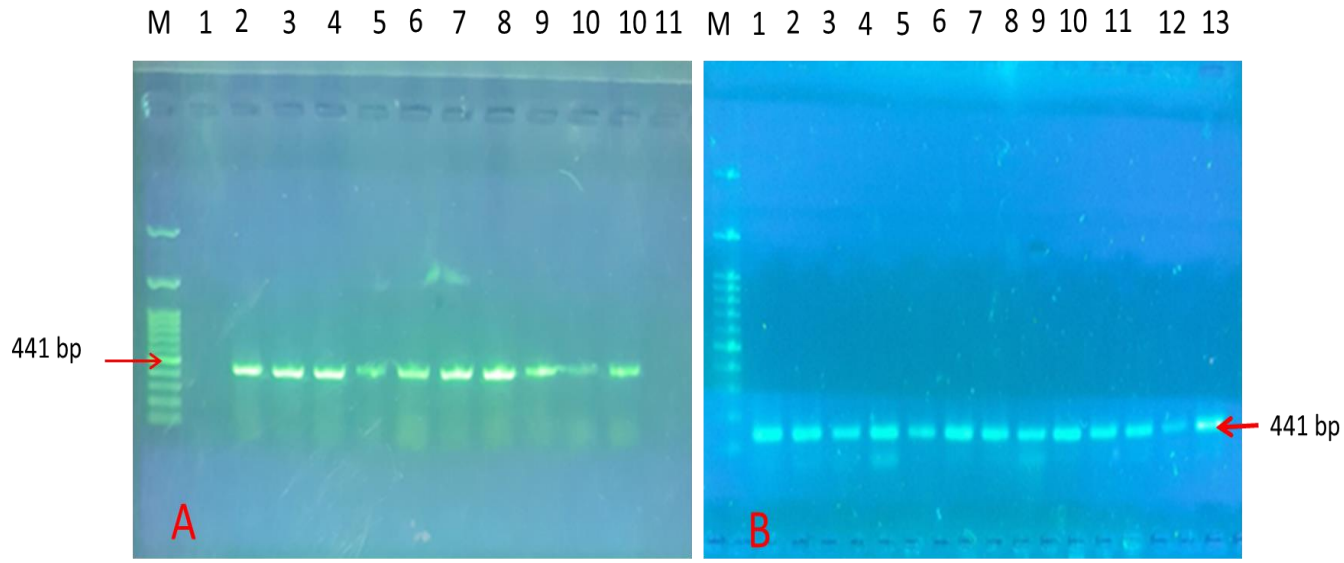

Fig 2. Panel (A) PCR amplification of gyrA (441) bp of *K. pneumonia*, lanes 1 and 11 represented negative samples, lanes 2-10 represented positive samples, Panel (B) PCR amplification of uidA (147) bp 457 of *E. coli* where lanes 1 -13 represented positive samples. Molecular weight marker: M=pBr322/Msp 1.5% agarose gels and stained with Safe dye.

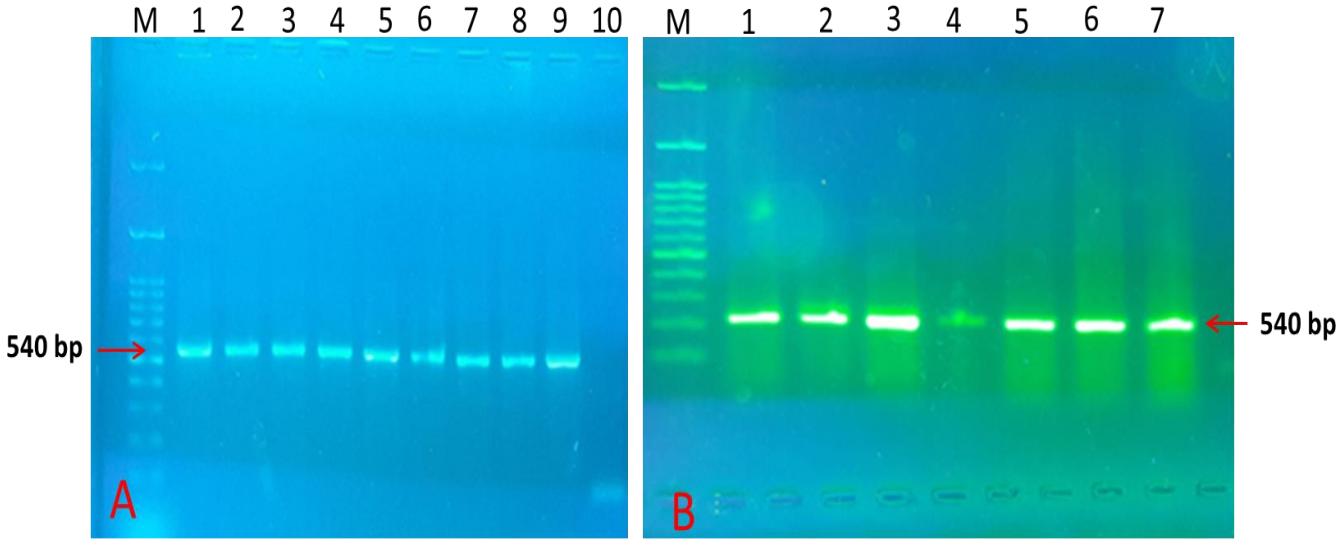

Fig 3. Panel (A) PCR amplification of zapA (540) bp of *Proteus* spp, lane (10) negative sample, :lanes 1-9 represented positive samples, Panel (B) PCR amplification of O-antigen acetylase (232) bp 457 of *Pseudomonus* spp Lanes from 1 -7 represented positive samples. Molecular weight marker: M=pBr322/Msp 1.5% agarose gels and stained with Safe dye.

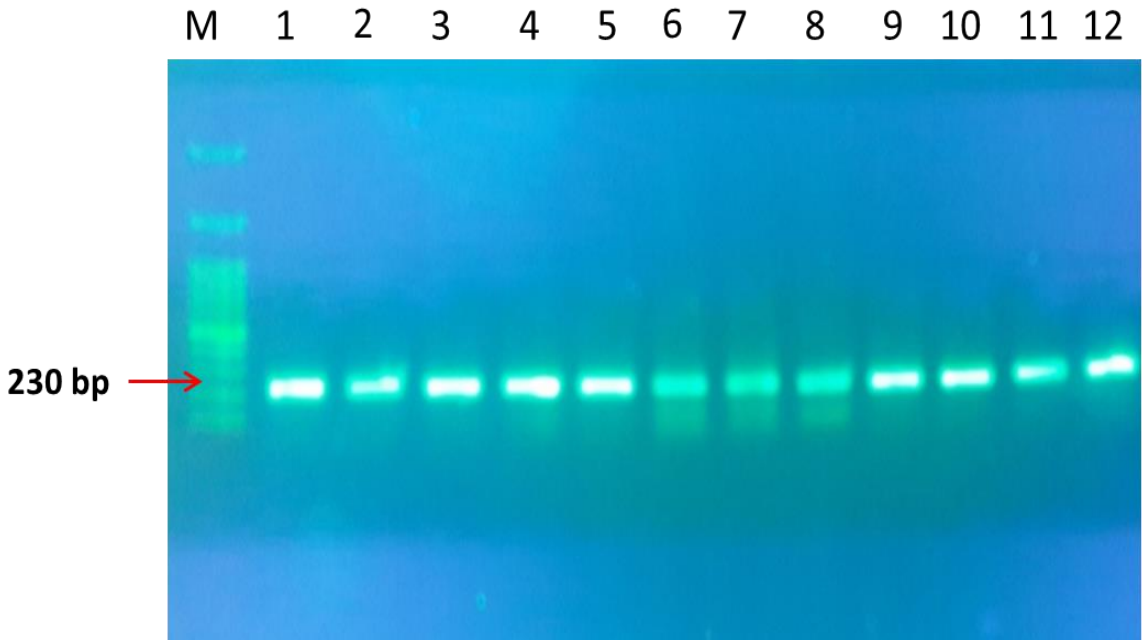

Fig. 4 PCR amplification of sod C (230) bp of *M. haemolytica*, Lanes 1-12 represented positive samples. Molecular weight marker: M=pBr322 /Msp 1.5% agarose gels and stained with Safe dye.

The variance in the prevalence rates of various types of pneumonia in small ruminants in different studies may be attributed to the variation in the factors such as nutritional status, breeding, nature of the country, and environmental conditions. In addition to the effect of stressors including transportation and overcrowding, the majority of these factors could possibly play a productive role in predisposing factors for the evolution of different types of pneumonia (Daniel et al. 2006; Ertan 2006).

In the current study, the isolation and identification of seven species of bacteria were confirmed by biochemical, staining characterization, and molecular techniques from pneumonic lung lesions of sheep and goat. Our finding observed that gram-positive bacteria were a higher portion than gram-negative. In sheep, *Staphylococcus aureus* (85%) was the major microorganism followed by E. coli (22%), *P. multocida* (6%), Mixed Bacteria *S. aureus* and *E. coli* (16%), *Klebsiella pneumonia* (4.8%), *M. haemolytica* (2,6%), and *Pseudomonous* spp (0.8%). The aerobic bacteria isolated from the pneumonic lungs with ones isolated by (Al –Anbagi 2016).

There are various studies near to our study for isolated bacteria from pneumonia, pathogenic lung lesions as well as normal flora of the respiratory system in sheep (Obi et al. 1983; Adekeye, 1984; Akpavie et al.1991).

In goat, Staphylococcus aureus (40%) was the main microorganism followed by *E. coli* (15%), Mixed Bacteria *Staphylococcus aureus* and *Escherichia coli* (13.33%), *P. multocida* (6%), *Klebsiella pneumonia* (4%), *M. haemolytica* (2,6%), and *Pseudomonous* spp. (2.6%). This study was in agreement with the finding by (Yesuf et al. 2012 ; Haque et al. 2007). *Staphylococcus* spp. inhabits in the upper respiratory tract and is involved in diseased processes only when stress circumstances prevail (Megra et al. 2006; Ferdausi et al. 2008) mentioned that the prevalence rate of Staphylococcus spp., Pasteurella spp., and Bacillus spp. were 36.67%, 11.67%, and 3.33%, respectively.

While the results of this study disagreed with (Ugoch et al. 2017) who reported above 55 % of the pneumonic lung samples was positive for E. coli which is more than the other isolated aerobic bacteria. There are numerous studies comparable to tstudy for bacteria isolated from pneumonic lung, pathogenic lesions as well as normal flora of the respiratory system in sheep (Obi et al.1983; Adekeye 1984; Akpavie et al.1991).

The variation in incidence percentage of causative agents of pneumonia in small ruminants due to the geographical area and several factors including different isolation processes, misidentification, stress, changes in management, transportation, immune state of infected animals, and seasonal variation (Naglaa et al. 2019; Azizi et al. 2013).

Our results agreed with study conducted by (Naglaa et al.,2019) and they showed that the percentage of *P. multocida* was more than that of *M haemolytica* and this may attributed to fact that the P. multocida is more related to the nasopharynx infection in acute cases more than *P. multocida* which related lung and *M. haemolytica* infection is also more common when the case is complicated by other microorganisms including respiratory viruses and mycoplasma infection also it may be attributed to the type of the sample that was only from one origin.

The present work showed that PCR could be used for the detection of causative agents of pneumonia in small ruminants. However, the culturing is more time-consuming, while PCR is a molecular technique for the detection of infectious agents and potent methods of directly recognizing these pathogens and epidemiological purpose, an excellent tool, the simple and quick method in clinical samples. PCR due to its offers' greater facility of use in comparison to cultures techniques related to biochemical tests for the identification of bacteria. In addition, the present study agreed with (Dag 2018; Sabiel, et al. 2012; Mohammad 2018) who indicated that PCR is very useful and time-saving for the identification of many of bacterial species causing pneumonia in ruminants especially *P. multocida* and *M. haemolytica*. Moreover, it has been showed that PCR is more accurate than the other technique for the identification of *Pasteurella* spp. (Tabatabaei et al.2018) Another study added that molecular techniques have a grand worth in the detection and typing of the different strains belonging to the family *Pasteurellaceae* (Beker et al.2018).

Conclusion

The study revealed a number of bacterial pathogenic pathogens associated with pneumonia in goats and sheep slaughtered over a period of one year (September 2020 to September 2021) at Duhok abattoir. The identified and isolated different types of bacterial species through agar morphology and biochemical characterization studies, beside PCR technique plays a confirmative role and is more accurate than conventional bacteriological methods in clinical laboratories for faster analysis of infectious diseases. Moreover, it is a reliable and sensitive complement to existing diagnostic tools.

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Conflict of interest

The authors declare that there is no conflict of interest.

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