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Histopathological and Molecular Investigation of *Pasteurella multocida* Specific Outbreak in a Sheep Flock with High Mortality in Egypt

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ABSTRACT

Pasteurella is a major bacterial pathogen causing respiratory signs due to pneumonia and septicemia that usually affects young animals and humans, leading to significant economic losses. it is responsible for highly contagious infections in animals, leading to high mortality and consequently financial losses for the breeders. The aim of the study was the rapid detection of *Pasteurella* infection outbreak. This study examined a specific outbreak of pasteurellosis in a sheep flock in Egypt based on clinical and postmortem diagnoses, molecular detection using universal and specific primers, as well as the histopathological examination to demonstrate the pathological changes on internal organs. The results revealed that 15 of 70 animals (21.4%)—primarily lambs under one year—died suddenly with mild respiratory signs. Postmortem examination revealed congested, consolidated lungs and liver lesions. Bacteriological culturing showed characteristic non-hemolytic colonies on the blood agar, and the Gram's staining confirmed bipolar coccobacilli. PCR targeting the 16S rRNA gene and SYBR Green real-time PCR targeting the *KMT1* gene identified *Pasteurella multocida* (*P. multocida*) in 8/10 samples. Septicemic bronchopneumonia and hepatic degeneration highlight the importance of histopathological investigation align with molecular assays for accurate diagnosis of *P. multocida* infection in sheep.

Keywords: *Pasteurella*, PCR, pneumonia, septicemia, lamb, Egypt

INTRODUCTION

Ruminants are an essential component of livestock production and serve as a major source of animal protein and income for smallholder farmers (**1**). Consequently, diseases affecting these animals have substantial economic repercussions for both farmers and national economies (**2**). Pneumonia is among the most common respiratory diseases in small ruminants worldwide. Moreover, pneumonic pasteurellosis being an acute infectious condition that causes significant financial losses due to mortality, reduced weight gain, and treatment costs (**3**). *Pasteurella* species are commonly detected in small ruminants (**4,5**), with Mannheimia haemolytica and *Pasteurella multocida* (*P. multocida*) as major causative agents for mannheimiosis and pasteurellosis, respectively. These microorganisms are found in pneumonic compared to healthy animals. *Pasteurella* are facultative anaerobic, nonmotile, and non-spore-forming, β-hemolytic coccobacilli or rods that may grow weakly on MacConkey's

agar. *P. multocida* does not have the ability to cause hemolysis on the blood agar. Moreover, they have greyish-white, smooth, flatly pointed colonies with a typical bleach odor (**7**).

Septicemic pasteurellosis is caused by multiple serotypes of *P. multocida*. The disease typically progresses through three stages: an initial febrile phase, followed by respiratory involvement, and septicemia leading to sudden death. Once clinical signs appear, mortality approaches 100% (**8**). Transmission of *P. multocida* to domestic sheep is believed to occur through a close contact with domestic goats, particularly via nose-to-nose interaction (**9**). *P. multocida* is also classified as a zoonotic pathogen, in which humans can acquire the infection through direct contact with the infected animal and its secretions (**10**). The effect of the pathogen on human is chronic and acute, ranging from pasteurellosis, pneumonia, rhinitis, abscesses, meningitis, and hemorrhagic septicemia (**11**). In small ruminants less than two months of age, *P. multocida* infection often causes septicemia and systemic pasteurellosis, with clinical manifestations including dyspnea, lethargy, anorexia, and sudden death (**12**).

Sheep affected with *P. multocida* suffer from pneumonia characterized by pulmonary congestion and consolidation. The inability to diagnose ovine pneumonia reliably during ante-mortem examination and the lack of precise information on lesion sites hinder the development of effective control strategies (**13**). Also, polymerase chain reaction (PCR) has replaced traditional diagnostic procedures. Real-time PCR has multiple benefits, including high sensitivity, quick results, and consistent specificity (**14**). The 16S rRNA gene is a universal marker for bacterial identification, but the *KMT1* gene is particular to *P. multocida* (**15**).

Despite the complexity and limited understanding of the causes of sudden death in neonatal lambs, *Pasteurella* species are increasingly recognized as major causative pathogens. Therefore, the present outbreak investigation aimed to confirm *P. multocida* infection in a small ruminant flock through clinical assessment, postmortem examination, and laboratory confirmation using molecular and histopathological techniques.

Materials and Methods

Ethical Approval

The current study protocol was approved by Animal Care and Use Committee at Zagazig University, and the protocol number was ZU-IACUC/2/F/65/2025.

Animals Examination and Sample Collection

In the winter of 2024, a smallholder with a flock of seventy sheep (Ovis aries Linnaeus) of different ages (from one-month-old lambs to four-year-old adults) admitted to the Veterinary Medicine Faculty Clinic at Zagazig University in Egypt. The owner reported sudden deaths among young lambs raised with adult sheep, with mild signs such as nasal discharge and coughing appearing just one day before death. While 15 lambs had died within a few days, the owner brought a freshly dead lamb for examination and sought veterinary advice on disease management. At the clinic, a postmortem examination was performed on the submitted carcass, and tissue samples (infected lungs and livers with nasal swabs) were collected for laboratory diagnosis. A field visit to the flock was also conducted to clinically examine the remaining animals, with all parameters recorded according to Constable et al. (**16**).

Samples were collected from the infected lung and liver tissues of the freshly dead animal. Each organ was sectioned aseptically; one portion of each organ was fixed in 10% formalin for histopathological examination, and the other portions were used to isolate *Pasteurella*. The weight of the collected organs mainly depended on the type and the condition of the infected organ, which is proportionally related to the number of bacteria. To isolate *Pasteurella*, about 10-20 g were put in 50-100 mL of nutrient broth (Oxoid, UK). Additionally, four nasal swabs were obtained from in-contact sheep exhibiting depression, mild coughing, and mucopurulent nasal discharge and incubated for 24-48 h at 37°C to allow bacteria to grow, following the protocol of Dunbar et al. (**17**). After that, all samples were transported to the Zagazig Animal Research Institute, Sharkia province, for bacteriological and molecular analysis.

Bacteriological Examination

Bacteria were isolated from the nasal swabs, lung, and liver using Oxoid blood agar (5% sheep blood) and MacConkey's agar (Oxoid, UK). The plates were incubated at 37°C for 18-24 h before being inspected for bacterial growth. Bacterial detection based on Quinn et al. (**18**) who established criteria for characterizing colony morphology. Gram's staining was performed on suspected colonies in accordance with the methodology of Ozyildiz et al. (**19**), with additional smears generated from cardiac blood to assess bipolarity.

DNA Extraction and PCR Assay

DNA was extracted from the bacterial colonies isolated from 10 samples (nasal swabs of in contact-sheep, lungs, and livers of dead sheep) using the QIAamp DNA Mini Kit (Qiagen, Germany, GmbH

A universal bacterial 16S rRNA gene primer set was employed, consisting of the forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and the reverse primer 5′-CTTGTGCGGGCCCCCGTCAATTC-3′, which amplified a 1485 bp fragment as described by Lagacé et al. (**20**). A 25- µL reaction mixture containing 12.5 µL of 2× Emerald Amp Max PCR Master Mix (Takara, Japan), with 1 µL of each primer of 20 pmol/µL concentration, 5.5 µL of nuclease-free water, and 5 µL of DNA template were used. The reaction was performed in an applied biosystem 2720 thermal cycler. The amplification cycle consisted of primary denaturation step at 94°C/5 min followed by 35 cycles of 30 sec secondary denaturation at 94°C, 1 min primer annealing at 56°C/min, 1.2 min extension at 72°C and a final extension of 12 min at 72°C. Finally, the PCR products were separated on 1.5% agarose gel (Applichem, Germany,

GmbH) in 1× TBE buffer at room temperature, and ethidium bromide stain (0.5 µg/mL) (Sigma-Aldrich) was added and mixed carefully. A positive control was a previously confirmed *Pasteurella* isolate submitted to the Animal Health Research Institute reference laboratory, while nuclease-free water was used as a negative control. The gel was transferred to an ultraviolet cabinet and photographed using a gel documentation system (Alpha Innotech, Biometra) in order to view the DNA bands after electrophoresis at 100 V for 45 min.

Molecular Identification of *P. multocida* by Real-Time PCR

The eight bacterial isolates were further detected using real-time PCR targeting the *KMT1* gene of *P. multocida* with the forward primer 5′-ATCCGCTATTTACCCAGTGG-3′ and the reverse primer 5′-GCTGTAAACGAACTCGCCAC-3′, as described by Townsend et al. (**21**). DNA amplification was done using a Step One Plus™ apparatus from Applied Biosystems (Foster City, California, United States), and was performed in a 25 µL reaction mixture containing 12.5 µL of 2× QuantiFast SYBR Green PCR Master Mix (FastStart™ PCR Master, 04710436001, Roche), 2 µL of primers (20 pmol/mL) (Chromogen Company, South Korea), 5 µL of the DNA template, and 5.5 µL PCR water. Real-time PCR cycling conditions consisted of 40 cycles of denaturation at 95°C for 15 sec, followed by annealing for 30 sec of 60°C, and 30 sec of extension at 55°C. Following analysis of the melting curve and SYBR green fluorescence intensity, a threshold cycle (Ct) value less than 35 was regarded a positive result.

Histopathological Assay

Liver and lung samples were collected, preserved in a 10% buffered neutral formalin solution, dehydrated in a gradually increasing alcohol, cleaned in xylene, and then embedded in paraffin. A microtome (Leica RM 2155, England) was used to slice 5 µm thick paraffin sections. After that, the sections were regularly stained with hematoxylin and eosin (**22**).

Statistical Analysis

Analysis was performed using SPSS software (version 26, IBM Corp., Armonk, NY, USA). All data were analyzed descriptively. The clinical parameters were expressed as absolute numbers and percentages. Pearson Chi square test was applied to detect the significant differences between control and respiratory disorders groups with respect to age, appetite, vital signs (body temperature, respiration rate, heart rate), nasal discharge, ocular discharge, cough, recumbency, and sudden death. The *P*-value, degrees of freedom (df), and Chi-square statistic (χ^2) were computed for analysis. $P < 0.05$ was taken into consideration as the significant level.

RESULTS

Clinical and Necropsy Findings

A descriptive record of clinical parameters of the investigated flock is documented in **Table 1**. Significant differences were detected between control and respiratory disorders group at the level of appetite, vital signs (body temperature, respiration rate, heart rate), nasal discharge, ocular discharge, recumbency, cough, and sudden death rate. The epidemiological features of the disease showed that the mortality rate was 21.4% (15/70), and death occurred primarily in animals up to 1 year.

Table 1. Clinical parameters in healthy (no signs) and respiratory disorder cases within the investigated farm

Variables	Categories	Healthy n= 25 count (%)	Respiratory disorders count (%) (n= 45)	Pearson χ^2	df	*P*-value
Age	Adults	10 (40.0)	15 (33.3)	0.311	1	0.5770
	Lambs	15 (60.0)	30 (66.7)			
Appetite	Normal	20 (80.0)	0.0 (0.00)	55.48	2	<0.0001
	Inappetence	5.0 (20.0)	10 (22.2)			
	Anorexia	0.0 (0.00)	35 (77.8)			
Rectal temperature (°C)	Normal	25 (100)	5.0 (11.1)	51.85	1	<0.0001
	Fever	0.0 (0.00)	40 (88.9)			
Respiration rate (breaths/min)	Normal	25 (100)	4.0 (8.90)	54.98	1	<0.0001
	Polypnea	0.0 (0.00)	41 (91.1)			
Heart rate (min)	Normal	20 (80.0)	10 (22.2)	21.91	1	<0.0001
	Tachycardia	5.0 (20.0)	35 (77.8)			
Nasal discharge laterality	Absent	25 (100)	0.0 (0.00)	70.00	2	<0.0001
	Unilateral	0.0 (0.00)	25 (55.6)			
	Bilateral	0.0 (0.00)	20 (44.4)			
Nasal discharge type	Absent	25 (100)	0.0 (0.00)	70.00	2	<0.0001
	Mucoid	0.0 (0.00)	18 (40.0)			
	Mucopurulent	0.0 (0.00)	27 (60.0)			
Ocular discharge	Absent	25 (100)	25 (55.6)	15.56	1	<0.0001
	Present	0.0 (0.00)	20 (44.4)			
Recumbency	Absent	25 (100)	30 (66.7)	10.61	1	0.001
	Present	0.0 (0.00)	15 (33.3)			
Cough	Absent	25 (100)	25 (55.6)	15.56	1	<0.0001
	Present	0.0 (0.00)	20 (44.4)			
Sudden death	Absent	25 (100)	30 (66.7)	10.61	1	0.001
	Present	0.0 (0.00)	15 (33.3)			

At necropsy, the main characteristic findings were observed in the lungs and liver. Pneumonic lungs were congested and firm texture, with rib impressions appearing on lung surfaces. The trachea was red in color and contained exudate, also a thickening and redness of intra-lobular septa of the lung was observed. The livers of morbid sheep showed congestion, petechial hemorrhages and thickening in texture (**Figure 1**).

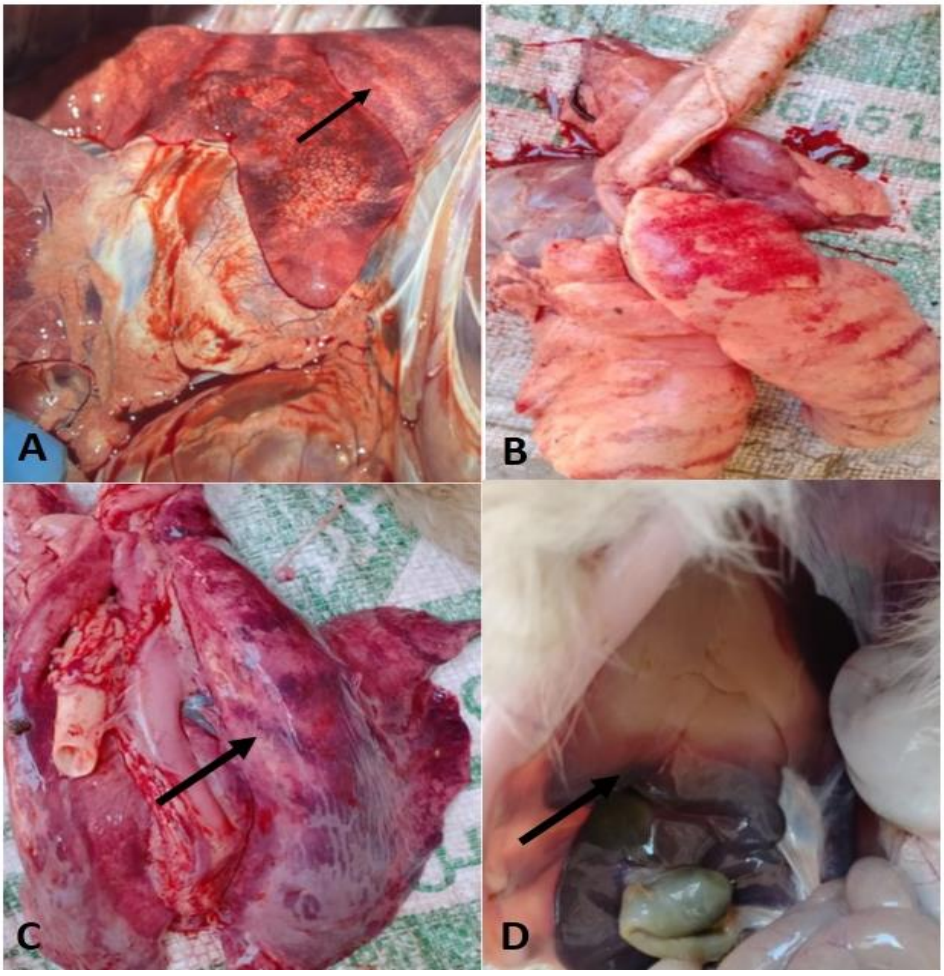

Figure 1. Gross lesions of sheep lungs and liver suspected to be infected with *Pasteurella*. The lung tissues **(A, B, C)** display rib impressions, thickening of the intra-lobular septa, and congestion. Liver **(D)** appears pale with congestion at its apical lobe

Identification of *Pasteurella*

Bacteriological examination of nasal and tissue swabs revealed round, white, non-hemolytic colonies on the blood agar, with no significant growth on the MacConkey's agar. These findings were observed in 8 of 10 samples (80%). Blood smear examination from heart blood demonstrated bipolar coccobacilli consistent with *Pasteurella* species. Molecular diagnosis showed the presence of a bacterial pathogen in eight culture isolates whose DNA extract was subjected to conventional PCR using universal 16S rRNA primers, producing a 1485 bp amplicon (**Figure 2A**) consistent with the positive control. Further confirmation of *P. multocida* occurred upon using SYBR Green real-time PCR targeting the *KMT1* gene, which identified all eight isolates as *P. multocida*, with amplification curves showing threshold cycle (Ct) values of 24, 26, and 28 (**Figure 2B**).

Histopathology

Histopathological examination of the lung tissue revealed degeneration and necrosis of the bronchial epithelium, consistent with necrotizing bronchopneumonia, accompanied by intrabronchiolar exudates composed of fibrin threads and erythrocytes. In addition, emphysematous areas and intra-alveolar fibrin deposits were evident (**Figure 3**). Furthermore, the pulmonary sections demonstrated marked vascular congestion and the presence of sero-fibrinous exudates within some alveolar lumina (**Figure 4**).

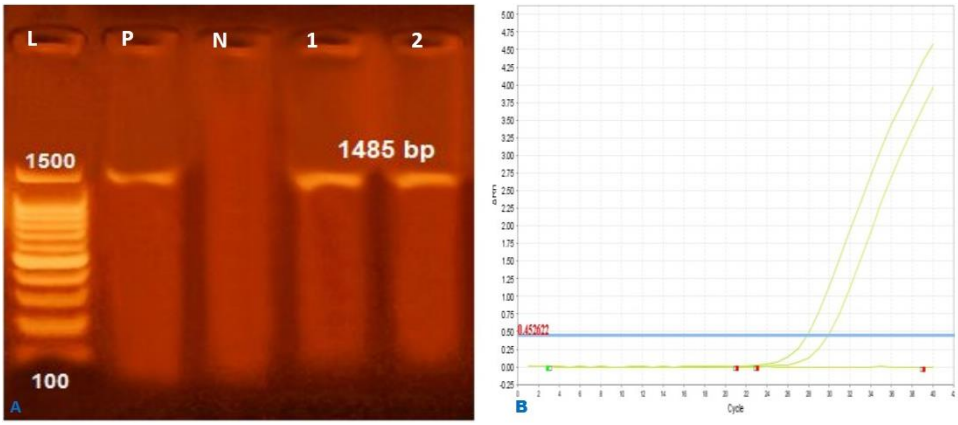

Figure 2. Molecular detection of *P. multocida*. (**A)** Agarose gel analysis of the PCR products from amplification of 16S rRNA. L: DNA ladder, P and N: Positiveand Negative control, respectively, 1and 2: positive bands at 1485 bp. (**B)** Amplification plot of the real time PCR targeting the *KMT1* gene of *P. multocida*

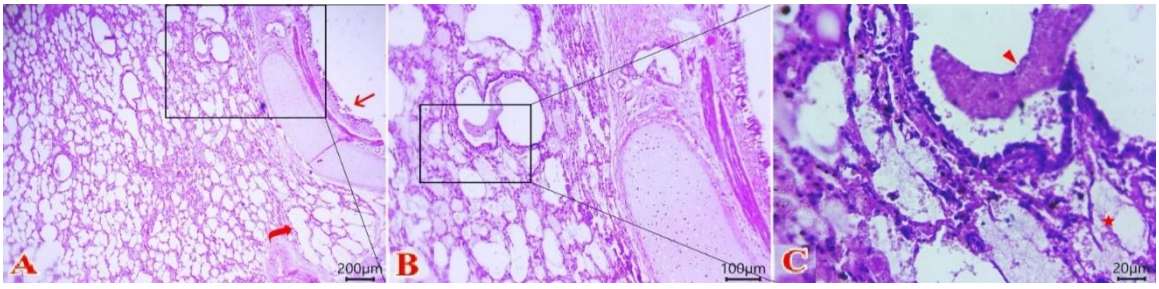

Figure 3. Photomicrographs of H&E-stained sections from lung showing: **(A, B, C)** some destructed bronchial epithelia (arrow), intrabronchiolar fibrin threads and erythrocytes (arrowhead), emphysematous area (curved arrow) and intra-alveolar fibrin threads (star). Scale bar 200, 100, and 20 µm

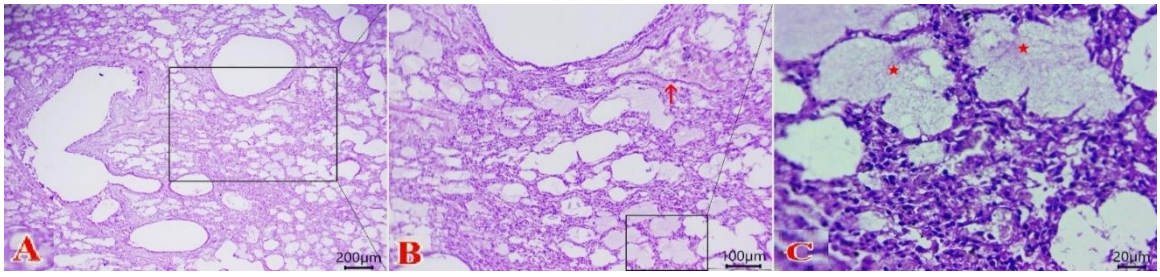

Figure 4. Photomicrographs of H&E-stained sections from lung showing: **(A, B, C)** congested pulmonary blood vessel (arrow), and sero-fibrinous exudate within some alveolar lumina (stars). Scale bar 200, 100, and 20 µm

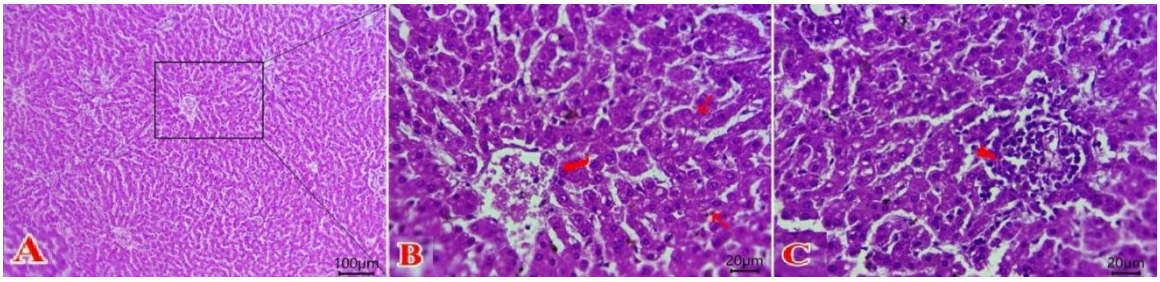

Figure 5. Photomicrographs of H&E-stained sections from liver showing: (**A, B, C**) intense vacuolar degenerations (arrows) in numerous hepatic cells, congested hepatic blood vessel (curved arrow), and focal areas of neutrophils infiltrations (arrowhead)

Similarly, liver tissue exhibited septicemic changes characterized by severe cellular degeneration, particularly vacuolar degeneration of hepatocytes. Moreover, congested hepatic blood vessels and focal neutrophilic infiltrations, mainly in perivascular regions, were observed (**Figure 5**). Additional findings included hyalinization of vascular walls, intravascular fibrin threads, red blood cells, leukocytic infiltration, and hemosiderin-laden monocytes (**Figure 6**). Finally, some portal areas showed fibrous connective tissue proliferation with hyalinized material, whereas bile ducts displayed cholestasis and epithelial hyperplasia (**Figure 7**).

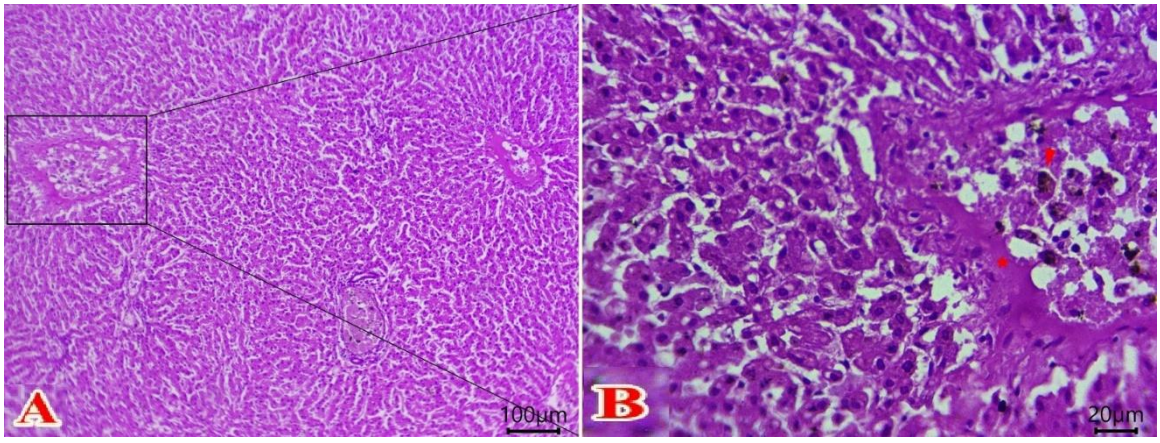

Figure 6. Photomicrographs of H&E-stained sections from liver showing: **(A, B)** hyalinized vascular wall (star) with intravascular fibrin threads, RBCs, some leukocytic infiltration and hemosiderin laden monocytes (arrowhead). Scale bar 100,20 µm

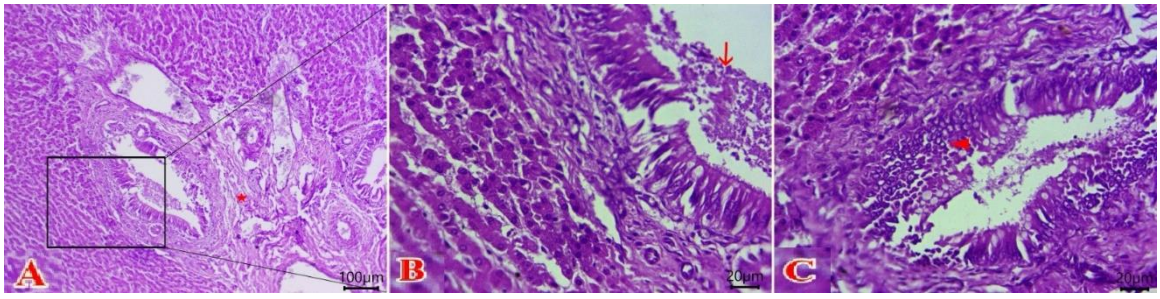

Figure 7. Photomicrographs of H and E-stained sections from liver showing: (**A, B, C)** thickening of portal area by fibrous connective tissue proliferations with hyalinized material (star), besides cholestasis (arrow), and hyperplasia of some bile ducts epithelia (arrowhead). Scale bar 200, 100, and 20 µm

DISCUSSION

In Egypt, sheep represent an important livestock species that help offset the shortage of cattle and buffalo meat production while also providing wool for the textile industry (**23**). *P. multocida* is an economically significant respiratory pathogen that interacts with host tissues, causing severe pathological changes and premature death in various animal species, resulting in substantial financial losses (**5, 24**). The present study aimed to diagnose ovine pasteurellosis through postmortem and microscopic examinations, molecular techniques, and histopathological evaluation of the affected tissues.

The current investigation reported that infected sheep exhibited severe coughing, mucopurulent nasal discharge, fever, and congested mucous membranes. Bahr et al. (**25**) and Chandrashekar et al. (**26**) concluded that severe systemic disorders are recorded during pneumonia in small ruminants. During infection, gross signs of pneumonia, thickening in interlobular septum and consolidated regions were detected in the lungs. Also, the liver was hard and had petechial hemorrhages. Our findings agreed with pathological alterations reported in previous studies (**27,28**). The *KMT1* gene is a reliable marker for identifying and characterizing *P. multocida* in sheep. This gene encodes for an outer membrane protein that is a strain-specific, allowing for the reliable detection of *P. multocida* (**29–33**).

SYBR Green-based quantitative PCR is a sensitive and analytical method that provides quick findings and has a good association with target copy number and cycle threshold (Ct) values. Eight samples were confirmed as *P. multocida* using *KMT1*-specific real-time PCR, while two nasal samples showed no amplification. The negative nasal swab samples may be due to several reasons, including the possibility of lower bacterial colonization compared to tissue samples, the presence of a non-bacterial infection, in

adequate deeply swabbing during collection of nasal samples, or faults in the transfer and preservation of the samples. The positive result of this study was agreed upon by other researchers (**33, 34**) who found that detecting *P. multocida* using 16S rRNA and *KMT1* gene targets is crucial for applying preventive strategies in the small ruminants flocks. Furthermore, molecular methods provide an accurate tools for identifying bacteria compared to the standard traditional methods.

Septicemic bronchopneumonia with intrabronchial pneumonia were observed during the histopathological examination. Our results align with other authors (**27,28,35**), who reported that pulmonary infiltrates of neutrophils, fibrin, proteinaceous material with extensive parenchymal necrosis, are likely attributable to *P. multocida* endotoxins. Emphysematous changes and intra-alveolar fibrin deposition were also observed in the current study, while pulmonary secretions demonstrated congested blood vessels and fibrinous exudate within alveolar lumina. Thacker (**36**) as well as **Müller and Köhler (37)** reported that neutrophil infiltration in pulmonary tissues reflects their innate immune role in phagocytosis and cytokine production, facilitating bacterial clearance from lung tissue. The gross and microscopic lesions observed in our study indicate vascular damage associated with septicemia and inflammation, as similarly reported by Chandrashekar et al. (**26**).

The liver's histopathological analysis showed a significant hepatocyte degeneration, and the blood vessels displayed hyalinization, congestion, and localized neutrophilic infiltration with intravascular fibrin threads. In a certain portal area, fibrous connective tissue was proliferated. Additionally, bile ducts showed signs of epithelial hyperplasia and cholestasis. The findings of Chandrashekar et al. (**26**) and Singh et al. (**33**) are in line with these lesions.

We concluded that *P. multocida* was the cause of sudden death in lambs suffering from septicemic pneumonia. Our investigation highlighted the value of histopathological and molecular tools for confirming *Pasteurella* infection in sheep. Also, Real-time PCR remains the gold standard diagnostic method compared with traditional bacteriological methods. Strict flock vaccination protocols, improved management strategies, and accurate diagnosis are highly recommended to reduce the risk of future outbreaks. Investigation of a single outbreak at a smallholder farm with a small sample size represents a certain limitation in our study; therefore, the results should be treated with caution. Future studies are highly recommended to confirm our findings.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, Methodology, Investigation, Resources, Writing – Original Draft, Writing – Review & Editing: EBA and MHE.; Writing – Review & Editing, Investigation, Resources: EBA, MBS, AIZ and MHE. All authors have read and approved the final version of the manuscript.

ARTIFICIAL INTELLIGENT DECLARATION

The authors declare that they are responsible for the accuracy and integrity of all content of the manuscript, including part generated by AI, and it is not used as a co-author.

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التحقيق الجزيئي والمرضى لجرثومة الباستريلا ملتوسيدا في قطيع من الأغنام ذو معدل وفيات مرتفع في مصر

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الخلاصة

تعتبر الباستيريلا واحدة من ضمن الأمراض البكتيرية التى تسبب مضاعفات كثيرة للحيوانات والأنسان بالإضافة الى خسائر اقتصادية كبيرة. فقد أستهدفت هذه الدراسة الى بحث تفشى المرض فى قطيع من الأغنام بمصر والتشخيص السريع للحد من انتشار المرض كواحدا من الأمراض سريعة الأنتشار بين الحيوانات. تمت هذه الدراسة على فحص تفشيًا محددًا لمرض الباستيريلوز في قطيع من الأغنام في مصر أعتمادا على التشخيصات الظاهرية والتشخيصات بعد الوفاة، بالإضافة الى التشخيص الجزيئي بإستخدام تفاعل البلمرةالمتسلسل باستخدام الجينات العامة والخاصة، كما تم الفحص النسيجي لأظهار التغيرات المرضية للمرض وتأثيره على الأعضاء الداخلية. أظهرت خمسة عشر حالة—معظمها من الحملان التي تقل أعمارها عن سنة— من أصل ٧٠ حيوانًا بالموت بشكل مفاجئ مع علامات تنفسية خفيفة بنسبه وفيات (٢١,٤٪). أظهر الفحص الظاهرى للحيوانات المتوفية أن الرئة محتقنة ومُتصلبة بالأضافة الى آفات في الكبد. أظهر الفحص البكتيرى على أجار الدم مستعمرات من البكتيريا غير مدمرة للدم، وأكد صبغ الجرام وجود كوكوباسيلات ثنائية القطب. بالأضافة الى التحقق الجزيئى للميكروب باستخدام تفاعل البلمره المتسلسل وثبت ايجابية العينات المفحوصة، وقد تم تحديد الجين المستهدف لبكتيريا الباستريلا ملتوسيدا حيث وجدت أيجابية ثمانية من عشرة عينات لكلا من الرئه والكبد.أوضحت نتائج الفحص النسيجى التهاب القصبات الهوائية الرئوي الإنتاني مع إفرازات ليفينية، واحتقان وعائي، وتغيرات تنكسية في الكبد. وفى النهاية تم أستنتاج أن التشخيص المبكر والتشخيص الجزئيى لبكتيريا الباستريلا ملتوسيدا خاصة فى الوقت المناسب ضرورى للحد من الخسائر الأقتصاديهة في قطعان المجترات الصغيرة. كما يجب التحسين والسيطرة على المرض بأستخدام اللقاحات الخاصه به، ومراقبة الحيوان والعلامات التنفسية.

الكلمات المفتاحية: الباستيريلا، تفاعل البلمره المتسلسل، التهاب رئوي، تسمم الدم، حمل، مصر