



Microbiological and Histopathological Study of Enterotoxemia Caused by *Clostridium perfringens* in Syrian Lamb

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ABSTRACT

Clostridium perfringens (*C. perfringens*) is one of the most common pathogens affecting sheep, causing significant economic losses due to high mortality rates. The aim of this study is to isolate and identify *C. perfringens* from lambs that had recently died from enterotoxemia and study the histopathological changes in their intestines, livers and kidneys. A total of forty-eight samples (16 swabs from each of the intestines, liver, and kidneys) were collected from the lambs. All the collected swabs from the internal cavity of the small intestine, liver or kidney were placed in sterile tubes prepared with thioglycolate broth. Tissue samples were also taken from the intestine, liver and kidney and placed in 10% formalin (NBF) containers. The samples were transferred to the scientific research unit at the College of Veterinary Medicine, Hama University for bacteriological and histological testing. All swabs underwent bacteriological examinations by both conventional and molecular techniques for the detection of *C. perfringens*. The *cpa*, *cpb*, *etx*, and *ia* genes were also investigated. The total isolation percentage was 79.16%, and the highest isolation percentage was recorded in intestine followed by liver and kidneys at 33.33%, 22.92%, and 22.92%, respectively. The study also revealed the presence of *cpa*, *cpb*, *etx* and *ia* genes at rates of 44.73%, 21.05%, 34.21%, and 5.26% among isolates. The results of the histopathological examination revealed that all the intestinal sections, liver and kidneys had various pathological lesions. This study provides an opportunity to know the pathogenic types in lambs that died as a result of *C. perfringens* infection and could serve as a useful control element for the development of vaccines to reduce and control this economic loss in lambs.

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INTRODUCTION

Clostridial enterotoxemia is an acute and often fatal disease that affects sheep of all ages, characterized by high and sudden mortality (Simpson *et al.*, 2018). It results from the absorption of bacterial toxins and leads to blood hemolysis. Toxins produced by *C. perfringens*, especially types A, B, C, D, and E, are considered the main causes of the disease (McDonel, 1980; Navarro and Uzal, 2019; Prescott, 2022). Enteric poisoning in sheep is primarily caused by infection with *C. perfringens* type D (Brown *et al.*, 2007). The prevalence of the disease in sheep ranges from 24.13 to 100% (El Idrissi and Ward, 1992; Greco *et al.*, 2005).

Enterotoxaemia results from several factors, including environmental or husbandry factors, such as sudden feed changes, overeating, and excessive consumption of high-protein, energy-rich foods, or

when animals consume large amounts of carbohydrates or a high percentage of carbohydrates in the feed, as well as excessive grazing, where it causes a slowdown of the intestinal movement (peristalsis) and stagnation of intestinal contents. With the presence of *C. perfringens*, coexisting in the large intestine, providing a suitable environment for the growth and reproduction of the bacteria, they continue to grow rapidly and excessively, allowing them to proliferate and spread to the small intestine associated with the production of large quantities of toxins, which will be absorbed into the blood and intestinal toxemia occurs (Quinn *et al.*, 2003; Nazki *et al.*, 2017; Simpson *et al.*, 2018). The disease has been reported in infant lambs that have delayed secretion of trypsin enzymes (Hassanein *et al.*, 2017; Prescott, 2022).

Other predisposing factors include decreased natural immunity, diseases that slow down bowel

movement, stress, and severe parasitic infections of the intestinal tract, such as those caused by worms and coccidia (Quinn *et al.*, 2003). *Clostridium perfringens* belongs to the genus *Clostridium*, which includes 209 species. These bacteria are anaerobic, sporulating, non-iron-reducing bacilli that are Gram-positive, non-motile, and encased in a polysaccharide capsule. They are blood hemolytic and exist in spore form in the environment as well as in the intestinal tract of humans, domestic and wild animals. Most of their species have been distinguished by sequencing of 16S rDNA gene (Uzal *et al.*, 2022; Kiu *et al.*, 2023).

Clostridium perfringens produces approximately 17 to 18 exotoxins, some of which play a major role in causing the disease in sheep (Prescott *et al.*, 2016). Alpha toxin, Beta toxin, Epsilon toxin, Enterotoxin, Beta₂ toxin, and Theta toxin are the most highly toxic toxins. These toxins are characterized by their diversity, multiplicity, and different characteristics, which complicate control, prevention, and treatment efforts (Navarro and Uzal, 2019; Uzal *et al.*, 2022; Kiu *et al.*, 2023).

Enterotoxemia in sheep is characterized by jaundice and enlargement of the spleen and liver and may be accompanied by the presence of red urine in the urinary bladder due to blood lysis (hematuria). Histopathological changes include liver necrosis, splenic congestion, glomerulonephritis, nephrosis, edema, and pulmonary congestion (McDonel, 1980).

There are still varying concepts and opinions regarding the pathological features and lesions associated with enterotoxemia in sheep. This variability arises from the methods and techniques used in disease diagnosing (Uzal *et al.*, 2004; Uzal and Songer, 2008). Therefore, this study was conducted to isolate and identify *C. perfringens* from sheep that recently died from enterotoxemia, as well as to examine the histopathological changes in the liver, intestinal epithelial lining, and renal tubules.

MATERIALS AND METHODS

Study area

This study was conducted on an Awassi sheep herd (lambs) at the Center for Scientific and Agricultural Research in Hama Governorate in Syria during the period from October 15, 2021 to June 30, 2022. The herd was improved through hybridization and selection for productive traits, raised in a semi-open pen system with specialized nutrition.

Samples

Forty-eight swab samples (16 swabs from each of the intestines, liver, and kidneys) were collected from recently died lambs due to intestinal poisoning. Their

ages ranged from 15 days to 8 months, encompassing both sexes (6 males and 10 females). The swabs were taken from the internal cavities of the small intestine, liver, and kidneys and were placed in sterile tubes containing thioglycolate broth (HiMedia®/India). After anatomical examinations were performed, the macroscopic pathological changes were recorded. Tissue samples were also collected from the intestine, liver, and kidneys and placed in 10% formalin (NBF) containers. The samples were then transferred to the Scientific Research Unit at the College of Veterinary Medicine, Hama University, for bacteriological and histological testing.

Bacterial isolation

The thioglycolate broth medium tubes were incubated anaerobically at 37°C for 24 to 48 hours. After incubation, they were transferred to selective Sulphite Polymyxin Sulphadiazine Medium (SPS) (HiMedia®/India) (Kalender *et al.*, 2023) and incubated anaerobically at 37°C for an additional 24 to 48 hours. Following, the cultures were grown on blood agar (HiMedia®/India) and incubated anaerobically at 37 °C for 24 to 48 hours. Positive samples were then subjected to Gram staining and biochemical tests including oxidase, catalase, sugar fermentation (sucrose, lactose, glucose, and maltose), and indole production (Nazki *et al.*, 2017).

Molecular diagnosis of the isolates

Extraction of DNA

The high pure PCR template preparation kit from Roche Applied Science® (Germany) was used to extract DNA from the *C. perfringens* isolates. According to Ahsani *et al.* (2010), three colonies that were grown on SPS agar were suspended in 350 µL of STET buffer (100 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 5% X-100) in 1.5-mL microtubes. Then, 25 µL of lysozyme (10 mg/mL) was added to each solution and the content was mixed. Subsequently, the microtubes were placed in a boiling water bath for 40 seconds, then the bacterial lysate was centrifuged at 13,000 rpm for 15 minutes at room temperature in a refrigerated microcentrifuge. After that, the supernatant was poured into a fresh microcentrifuge tube, and the nucleic acids were precipitated from it by adding 40 µL of 2.5 M sodium acetate (pH 5.2) and 420 µL of isopropanol. Precipitated nucleic acids were recovered by centrifugation at 13,000 rpm for ten minutes at 4°C. Next, the supernatant was removed and the pellet of nucleic acid was rinsed with 1 mL of 70% ethanol at 4°C. Finally, the pellet was dried and resuspended in 50 µL of TE buffer containing RNase. The concentration of the extracted DNA was subsequently measured using the Genova Nano Jenway device from the United Kingdom. The extracted DNA was then stored at -20 °C until needed.

PCR Reaction

The PCR technique was employed to identify the *cpa*, *cpb*, *etx*, and *ia* genes of *C. perfringens*. A total volume of 30 µl was used for the PCR reaction, prepared in a 200 µl tube (Biozym, Oldenhof, Germany). The reaction mixture consisted of 15 µl of Promega Corporation's (2×) GoTaq Green Mix Master, 1 µl of

primer 1, 1 µl of primer 2, 9 µl of Promega Corporation's (USA) DNeasy-free water, and 4 µl of the *C. perfringens* DNA template. The resultant amplicons were analyzed using gel electrophoresis on a 2% agarose gel (Peqlab, Erlangen, Germany), with a 100 bp ladder as a reference. As shown in Table 1, the PCR technique was used to amplify the specific genes of the isolates and the corresponding programs.

Table 1: The primers used for PCR and molecular weight of *C. perfringens*.

Gene Specificity	primer	Sequence (5'-3')	Product size (bp)	PCR Program	Reference
<i>cpa</i> (Alpha toxin)	<i>cpa</i> F	GCTAATGTTACTGCCGTTGA	204	A	(Titball <i>et al.</i> , 2003)
	<i>cpa</i> R	CCTCTGATACATCGTGTAAG			
<i>cpb</i> (Beta toxin)	<i>cpb</i> F	GCGAATATGCTGAATCATCTA	236	B	(Hunter <i>et al.</i> , 1993)
	<i>cpb</i> R	GCAGGAACATTAGTATATCTTC			
<i>etx</i> (Epsilon)	<i>etx</i> F	GCGGTGATATCCATCTATTC	541	C	(Hunter <i>et al.</i> , 1992)
	<i>etx</i> R	CCACTTACTTGTCTACTAAC			
<i>ia</i> (Iota)	<i>ia</i> F	ACTACTCTCAGACAAGACAG	317	D	(Perelle <i>et al.</i> , 1993)
	<i>ia</i> R	CTTCCTTCTATTACTATACG			

PCR program was as follows: program A consisted of 35 cycles (94°C for 45 seconds, 51-57 °C for 58 seconds, and 72°C for 60 seconds); program B consisted of 35 cycles (94°C for 45 seconds, 46 to 49°C for 57 seconds, 72°C for 60 seconds); program C consisted of 35 cycles (94°C for 45 seconds, 46to 52°C for 54 seconds, 72°C for 60 seconds); and program D consisted of 35 cycles (94°C for45 seconds, 46 to52°C for 54 seconds, and 72°C for 60 seconds).

RESULTS

The initial results of *C. perfringens* isolation from various samples revealed a variation in isolation rates among different organs. Out of 48 samples, a total of 38 samples were isolated, resulting in an isolation percentage of 79.16%. The highest isolation percentage was recorded in the intestine, followed by the liver and kidneys at 33.33%, 22.92%, and 22.92%, respectively, as shown in **Fig.1**.

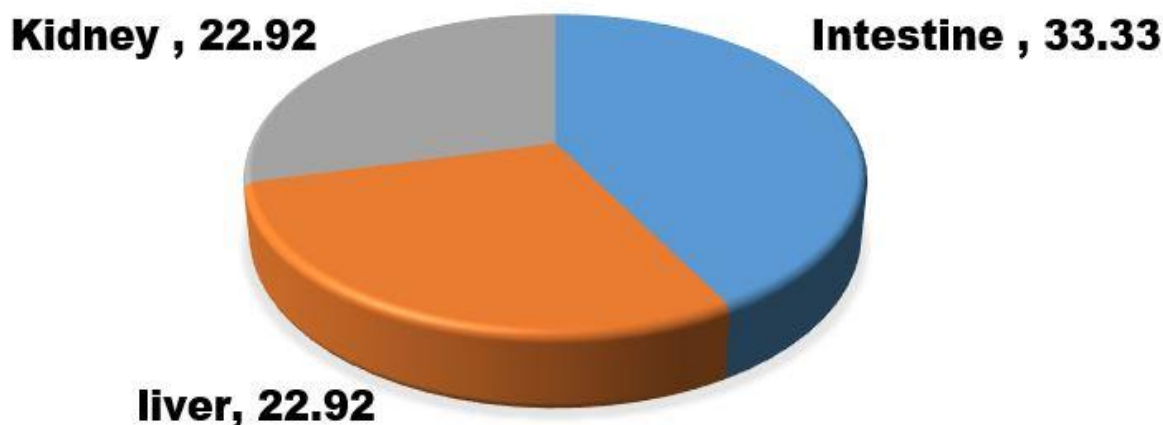


Fig.1: Total percentages of *C. perfringens* isolated from the different samples.

The results of bacterial isolation of *C. perfringens* on SPS agar indicated that the presence of medium to large, flat, circular, gray colonies. Microscopically, the isolates were Gram-positive bacilli arranged in chains, as shown in **Fig.2**.

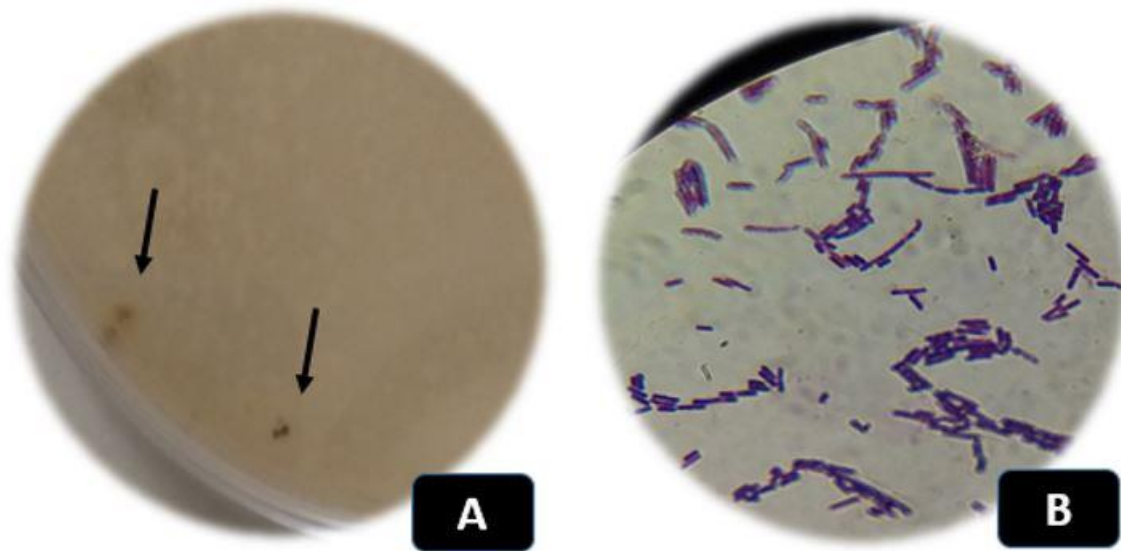


Fig.2: Phenotypic characterization of *C. perfringens* isolates; **A-** Growth of colonies on SPS agar, **B-** Gram staining smear (1000X).

The results of biochemical tests for *C. perfringens* isolates were negative for catalase, oxidase, and indole production but positive for sugar fermentation (glucose, mannose, sucrose, and lactose). The study also revealed that the presence of virulence genes for *C. perfringens*, which were evident from the results of amplification and electrophoresis on agarose gel, was indicated by the appearance of bands corresponding to the *cpa* gene at 402 bp, the *cpb* gene at 236 bp, the *etx* gene at 541 bp, and the *ia* gene at 317 bp. The prevalence rates were 44.73%, 21.05%, 34.21%, and 5.26%, respectively, out of 38 isolates from the intestine, liver and kidneys (**Table 2**).

Table 2: The percentage of the positive samples and the virulence genes of *C. perfringens* isolates

Samples	No. of +ve samples	% Virulence gene of isolates							
		<i>cpa</i>		<i>cpb</i>		<i>etx</i>		<i>ia</i>	
		No.	%	No.	%	No.	%	No.	%
Intestine	16	7	43.75	3	18.75	6	37.5	2	12.5
liver	11	5	45.45	2	18.18	4	36.36	0	0
Kidney	11	5	45.45	3	27.27	3	27.27	0	0
Total	38	17	44.73	8	21.05	13	34.21	2	5.26

The *cpa* and *cpb* genes were found in most isolates (from the intestine, liver and kidneys) at varying rates, while only two isolates, both from intestinal sources, carried the *ia* gene. The highest percentage of *cpa* and *etx* gene-carrying isolates were from intestinal sources, as shown in **Table 2**, **Fig.3**, **Fig.4**, **Fig.5** and **Fig. 6**.

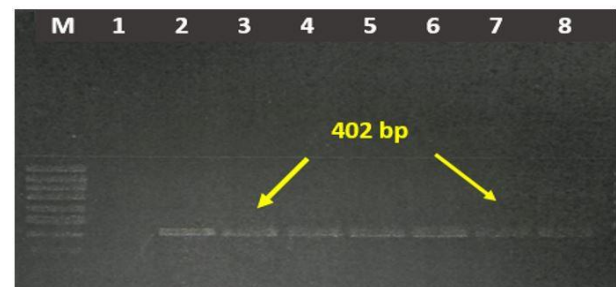


Fig.3: Typical amplification of *cpa* gene of *C. perfringens* with a molecular weight at 402 bp (M= Marker, 1= control negative, 2-8= Positive isolates).

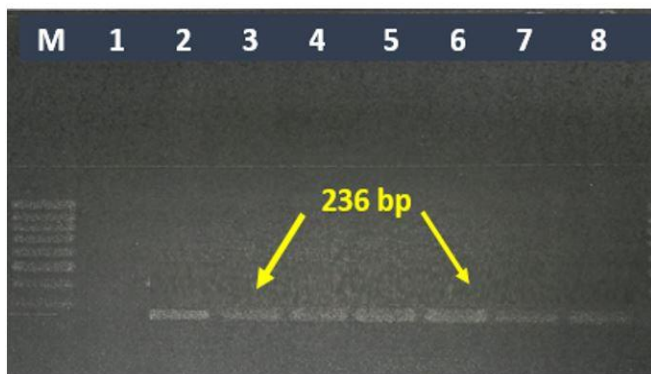


Fig. 4: Typical amplification of *cpb* gene of *C. perfringens* with molecular weight at 236 bp. (M= Marker, 1= control negative, 2 to 8= Positive isolate).

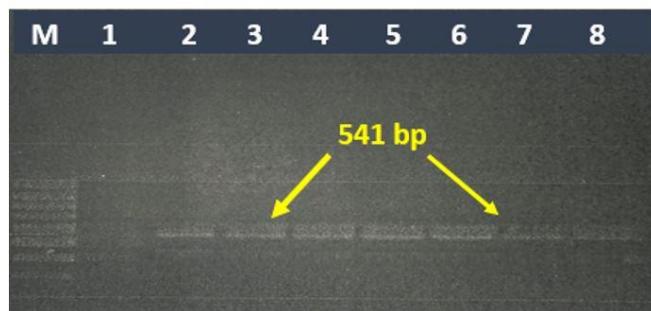


Fig. 5: Typical amplification of *etx* gene of *C. perfringens* with molecular weight at 541 bp. (M= Marker, 1= control negative, 2-8= Positive isolate).

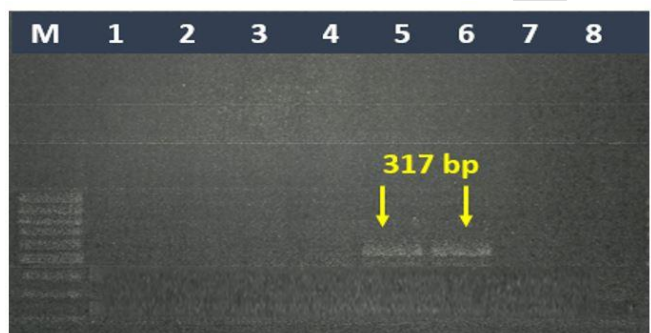


Fig. 6: Typical amplification of *ia* gene of *C. perfringens* with molecular weight at 317 bp (M= Marker, 1= control negative, 2-8= Positive isolate).

All slide sections were examined in detail and revealed that liver lesions included fatty degeneration and vacuolar degeneration, along with some hepatocellular nuclear condensation. Some atrophied sinusoid was observed with dilatation (**Fig.7**). Regarding the intestinal sections, there was massive infiltration of inflammatory cells, necrosis of epithelial cells was detected in some sections, desquamation of the epithelial layers of the crypts, and intestinal edema (**Fig.8**). In the kidney histopathological sections, there was dilatation of Bowman's capsule and necrosis of renal epithelium with cloudy swelling, forming a star

shape. Hyaline casts were seen in some renal tubules (**Fig. 9**).

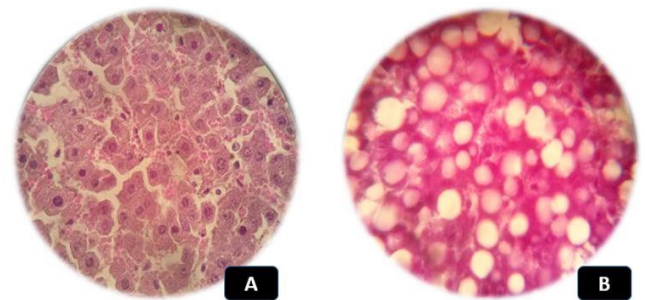


Fig.7: Histopathological sections of sheep liver (A) Hydropic degeneration, Hepatic sinusoids congestion (HandE) (100X). (B) Vacuolar degeneration (HandE) (400X).

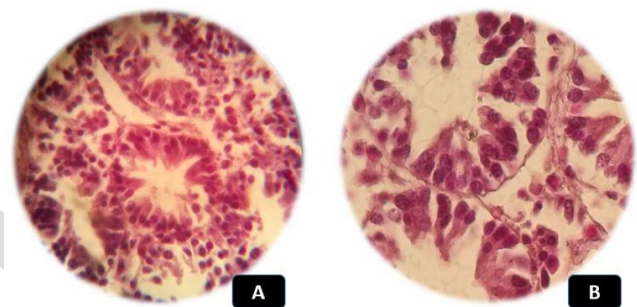


Fig.8: Histopathological sections of sheep intestine (A) Necrosis of intestinal mucosal layer – infiltration of inflammatory cells (HandE) (100X). (B) Degeneration and necrosis of intestinal villi and crypt (HandE) (400X).

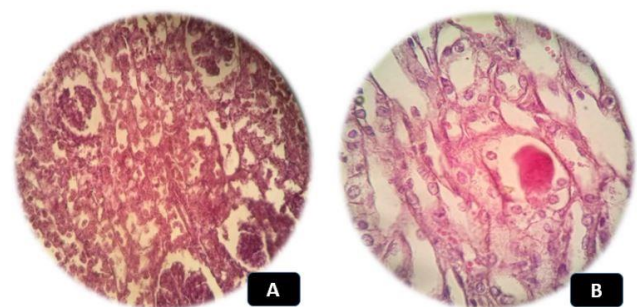


Fig.9: Histopathological sections of sheep kidney (A) Degeneration and necrosis of glomerular, dilatation of bowman's capsule (HandE) (100X). (B) Proteinaceous materials deposit in the renal tubules (HandE) (400X).

DISCUSSION

Enterotoxemia causes significant economic losses in fattening sheep and sheep raised on pasture (**Ikhomovich and Khaitovich, 2024**). Although there are vaccination programs at the Center for Scientific and Agricultural Research in Hama Governorate, in Syria,

they have unfortunately not been effective enough, leading to infections and deaths in the herds.

Bacterial colonies observed in anaerobic growth on SPS agar were identified as *C. perfringens* using conventional biochemical tests, as confirmed by **Saeid Hosseinzadeh et al., (2018); Kalender et al., (2023)**. Bacteriological examination of the samples showed the presence of *C. perfringens* in 79.16% of suddenly dead sheep.

The severity of the disease, whether caused by the agent itself or characterized as acute or per-acute, is consistent with what was indicated by many studies, including **Songer (1997) and Hassanein et al., (2017)**. In our study, the mortality rates ranged from 0-80% with an average of 40%, which differs from the findings of **Gökce et al., (2007); Tooloei and Masodei (2008)** where they indicated a 30% infection rate and a 100% mortality rate. Additionally, a study conducted in Syria by **Hamad (2010)** studied the percentage of dead cases in sheep flocks in the Hama city and its countryside, finding disease prevalence rates between 7.03% and 47.32%, with an average of 18.61%. This difference may be attributed to isolation methods and vaccination and prevention programs against the disease.

The percentage of bacterial isolation from the studied organs of dead animals was 33.33%, 22.92% and 22.92% in the intestine, liver and kidneys, respectively; these results align with the findings of **Vinod Kumar et al., (2014)**. The positive results of the small intestine culture indicate the presence of *C. perfringens* in its pathogenic form, while positive results from the liver, kidneys, or both confirm the infection. These microorganisms can enter the bloodstream after compromising the intestinal wall and subsequently spread to other organs (**Ali et al., 2024**)

Clostridium perfringens produces enterotoxins and lethal toxins, with each type of *C. perfringens* having its own specific set of toxins. These toxins are the basis for the clinical signs and pathological lesions observed in infected animals (**Assis et al., 2002**).

Modern molecular techniques have facilitated the identification of *C. perfringens* types by detecting the genes responsible for toxin production and by designing primers to distinguish between toxic and non-toxic types. A two-step method is recommended: after the PCR reaction with the primer mixture, PCR amplification can be performed using only the primers. In this study, types A, C, D and E were identified from intestinal isolates, and types A, C and D from liver and kidney isolates, as confirmed by **Diab (2016) and Uzal et al., (2022)**.

Clostridium perfringens type D produces epsilon toxin, which is resistant to digestive enzymes and converts protoxin into highly toxic epsilon toxins. These toxins are produced in the digestive system and absorbed into the circulatory system, leading to increased capillary permeability, which causes damage to the intestines, liver and kidneys, results in systemic bleeding in various organs (**Miyashiro et al., 2007; Elsify et al., 2016; Hassanein et al., 2017; Renu et al., 2022**).

Type A and C are the most common strains of *C. perfringens*, naturally found in the intestines of ruminants, particularly young lambs. They cause Yellow Lamb Disease and are characterized by their ability to induce hemolysis and lead to death in lambs (**Moustafa et al., 2022; Uzal et al., 2022**). This was confirmed by our study, which isolated *C. perfringens* from lambs of two months and younger.

Type E is the least common strain of *C. perfringens* identified by the detection of the *ie* gene, which is responsible for producing iota toxin associated with enteritis (**Sipos et al., 2003; Songer and Miskimins, 2004; Redondo et al., 2013**). In the current study, the *ie* gene was identified in two isolates obtained from older lambs.

The histopathological study results showed infiltration of inflammatory cell, necrosis of epithelial cells with desquamation of epithelial layers of the crypts, and intestinal edema in the intestinal sections. In the liver sections, there were signs of fatty infiltration, vacuolar degeneration with some hepatocellular nuclear condensation, and atrophied sinusoid accompanied by dilatation. Kidney sections revealed dilatation of Bowman's capsule and necrosis of renal epithelium, with cloudy swelling of the renal epithelium. These findings are consistent with **Mudendahangombe et al. (2006); Fernandez-Miyakawa et al., (2007); Radostits et al., (2007); Hussein et al., (2012); Nasir et al., (2013); Elsify et al., (2016); Hassanein et al., (2017)** who observed similar results when experimentally treating sheep with *C. perfringens*. They explained that the presence of receptors for toxin binding on the surfaces of red blood cells leads to hemolysis, tissue destruction, and the transfer of toxins through the blood to various organs, resulting in pathological lesions and death.

CONCLUSIONS

Despite the vaccination programs implemented against *C. perfringens* infections, their effectiveness in reducing cases remains limited due to the variation in clostridial types, high contamination rates, and the inadequacy of biosecurity measures. This study offers valuable insights into the pathogenic types of *C.*

perfringens associated with lamb deaths. Molecular characterization of *C. perfringens* strains using specific primers can serve as an essential tool for developing vaccines aimed at reducing and controlling the disease, which causes significant economic losses in lambs.

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors Contributions

The experiment was designed by the first and second researchers, while the other researchers contributed to the implementation of the work, analysis of the results, and writing the manuscript, including the financial costs.

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