Indian J Anim Health (2026)
DOI: https://doi.org/10.36062/ijah.2026.04625

Research Article

Isolation, phenotypic and biochemical characterization of *Clostridium perfringens* from healthy and diarrheic small ruminants

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Abstract

In the present study, 158 samples (rectal swabs and intestinal tissues) were collected from healthy and diarrheic sheep and goats from different areas in Krishna and West Godavari districts of Andhra Pradesh. Both rectal swabs and intestinal tissues were collected, and morphological, cultural and biochemical characters of all the isolates have been studied in detail. A total of 34 samples were found positive, indicating the prevalence of *Clostridium perfringens* to be 21.51% in the population. A higher isolation rate was obtained from intestinal tissues (67.65%) than rectal swabs (32.35%). Out of these isolates, 30 (88.23%) were characterized as *C. perfringens* type A, one (2.94%) isolate was characterized as *C. perfringens* type D and three (8.82%) isolates were characterized as *C. perfringens* type F by using multiplex PCR for six toxin genes, namely alpha, beta, epsilon, iota, entero and beta2. The sugars like galactose, cellobiose and raffinose were used to differentiate toxin type A, D and F from other toxin types, respectively. A representative number of isolates were subjected to antibiotic sensitivity test against commonly used ten antibiotics. They showed the highest resistance to sulphamethizole, followed by co-trimoxazole, enrofloxacin and tetracycline and high sensitivity observed against gentamicin, followed by cefotaxime/clavulanic acid, norfloxacin and chloramphenicol.

Keywords: Antibiotic sensitivity, Biochemical characterization, *Clostridium perfringens,* Isolation

Highlights

- *Clostridium perfringens* was isolated from healthy and diarrheic small ruminants.
- Morphological, cultural and biochemical characters were studied.
- Multiplex PCR was used for molecular characterization.
- Three toxin types i.e. *C. perfringens* type A, D and F, were isolated.
- Sugar fermentation test was used to differentiate toxin types.

INTRODUCTION

Diarrhoea is one of the major causes of mortality in small ruminants. Many of the agents are capable causing diarrhoea of which *Clostridium perfringens* were found to play an important role in inducing diarrhoea and heavy mortalities in small ruminants. Clostridial disease is associated with rapid bacterial overgrowth within the gastrointestinal tract and subsequent exotoxin release. *C. perfringens,* a rapid growing pathogen known to secrete more than 20 extracellular toxins and hydrolytic enzymes (Uzal et al., 2014). Toxins produced by *C. perfringens* types causes several histotoxic and enterotoxic diseases, including hemorrhagic enterocolitis, enterotoxaemia, pulpy kidney disease, over eating disease, struck, lamb dysentery, enterotoxaemic jaundice, yellow lamb disease, clostridial abomasitis, clostridial enteritis (Uzal et al., 2014). Currently, *C. perfringens* strains are categorized into seven toxin types (A to G) based on the genotypic expression of four major toxins, alpha, beta, epsilon and iota and two minor toxins, namely, enterotoxin and NET B toxin (Saadh et al., 2022). *Clostridium perfringens* type A produce α toxin; type B produce α toxin, β toxin and ε toxin; type C produce α toxin and β toxin; type D produce α toxin and ε toxin; type E produce α toxin and ι toxin; type F (formerly known as enterotoxin positive strains of *C. perfringens* type A) produce α toxin and enterotoxin (CPE); and type G produce α toxin and Net B toxin whereas β2 toxin found to be produced by any toxin type and CPE may also produced by type C, type D and type E.

Knowledge about the distribution of different toxin types in an area is important for recommending proper control measures. Since there is paucity of information on distribution of various toxin types in Krishna and West Godavari districts of Andhra Pradesh, the present study is planned on isolation, identification,

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biochemical and molecular characterization of *C. perfringens* with the objectives, to isolate *C. perfringens* from diarrhoeic and healthy small ruminants (sheep and goat); to identify *C. perfringens* by cultural, biochemical and molecular tests; and to detect and type the *C. perfringens* toxins by PCR.

MATERIALS AND METHODS

A total of 158 samples (rectal swabs and intestinal tissues) were collected from healthy and diarrheic sheep and goats of different age groups in different areas of Krishna and West Godavari districts of Andhra Pradesh. The details of samples collected were represented in Table 1 & Table 2.

Table 1. Details of samples collected

Animal	No. of samples collected		
	Rectal swabs		Intestinal
	Healthy	Diarrheic	tissues
Sheep	25	26	36
Lamb	3	33	8
Goat	3	6	3
Kid	2	8	5

Table 2. List of different samples collected from different areas of Krishna and West Godavari districts of Andhra Pradesh

Place	Diseased								Healthy			
	Sheep		Lamb		Goat		Kid		Sheep	Lamb	Goat	Kid
	RS	I/T	RS	I/T	RS	I/T	RS	I/T	RS	RS	RS	RS
Vijayawada	-	7	-	-	-	1	-	-	3	-	1	-
Kabela	-	6	-	-	-	-	-	-	-	-	-	-
Kesarapalli	-	4	2	-	-	-	-	-	2	-	-	-
Gannavaram	-	-	4	2	-	-	3	1	-	1	-	1
Nidakudhuru	1	-	5	-	-	-	2	-	1	1	-	1
Mudinepalli	6	-	2	3	-	-	-	-	3	-	-	-
Kaanukollu	3	-	4	3	-	-	-	-	2	-	-	-
Allapuram	3	-	-	-	4	-	-	-	2	-	1	-
Bandarugudem	-	6	-	-	-	-	-	-	3	-	-	-
Mylavaram	2	-	4	-	2	-	-	4	2	-	1	-
Challapalli	7	-	5	-	-	-	-	-	2	-	-	-
Jeelugumilli	4	9	7	-	-	2	3	-	5	1	-	-
Chinthalapudi	-	4	-	-	-	-	-	-	-	-	-	-

RS- Rectal swabs; I/T- Intestinal Tissues

Clostridium perfringens maintained in the Department of Veterinary Microbiology, NTR College of Veterinary Science, Gannavaram was used as the reference strain for the present study.

The swabs or intestinal tissues were suspended in sterile normal saline and heated in a water bath at 80°C for 20 minutes and then inoculated in Thioglycolate broth and incubated for 18-24 h at 37°C under anaerobic conditions. These samples were subjected to serial dilution with Buffered Peptone Water and spread on Perfringens agar base (T. S. C – Tryptose sulphite cycloserine agar) with egg yolk emulsion medium and incubated at 37°C for 36 to 48 h in McIntosh and Filde's anaerobic jar containing AnaeroGas Pak 3.5 L. After incubation, characteristic black colonies surrounded by opalescence were tested for Gram's staining and streaked on Perfringens agar base (T. S. C) added with egg yolk emulsion and incubated at 37°C for 36 to 48 h anaerobically. Gram's staining was performed by the standard method (Carter & Cole, 1990). A series of biochemical tests that includes Catalase, Oxidase, Gelatin hydrolysis, Stormy clot fermentation, Lecithinase, Sugar fermentation tests and Hemolysis pattern on 5% sheep blood agar were performed according to standard methods.

Nutrient gelatin was prepared as per the manufacturer's instruction and was stabbed with *C. perfringens* isolates with the help of straight inoculation wire and incubated anaerobically at 37°C for 7 – 14 days, with the incidence of liquefaction checked daily by chilling at 4°C for 10- 15 min along with control tube (Dela Cruz & Torres, 2012).

The litmus milk medium was prepared as per the manufacturer's instructions. *C. perfringens* culture was inoculated in the freshly prepared medium and boiled at 80°C for 10 min and then incubated anaerobically at 37°C for 24- 48 h and tested for the presence of stormy clot and gas production.

The test culture was streaked on egg yolk agar (EYA) plates prepared by adding the 10% egg yolk emulsion to the autoclaved molten nutrient agar at 45°C, mix it gently and set for solidification. Then the plates were incubated anaerobically at 37°C for 48 h and observed for the presence of opalescence around the colonies.

Blood agar was prepared by adding 5% aseptically collected defibrinated sheep blood to the autoclaved molten nutrient agar at 45°C and set for solidification following gentle mixing of the media. *C. perfringens*

isolates were streaked on blood agar plates and incubated anaerobically at 37°C for 48 h and were observed for the hemolysis pattern.

The samples that exhibited characteristic cultural and biochemical characters were subjected to PCR, and then the positive isolates were subjected to multiplex PCR fir toxin typing.

Sugar fermentation test for previously confirmed toxin types by multiplex PCR was performed as per the method of Kumar et al. (2014). For this, 5 mL of peptone water was taken in each test tube and Durham's tube was placed therein to detect the gas production and sterilized by autoclaving. One sugar disc was added to each tube aseptically. Each tube was inoculated with 0.1 mL of inoculum and incubated anaerobically at 37°C for 24 h. On addition of Andrade's reagent, the results of colour change were recorded as positive or negative. A control using 0.1 mL sterile water as inoculum was used to compare the colour change. Different sugars used were sucrose, lactose, rhamnose, arabinose, mannitol, trehalose, raffinose, cellobiose, galactose, and mannose.

Antibiogram of *Clostridium perfringens* isolates was carried out by the standard Kirby-Bauer disc diffusion method (Bauer et al., 1966). The *C. perfringens* was sub-cultured in Thioglycolate broth and incubated anaerobically for 24 h at 37°C. The turbidity of the bacterial suspension was adjusted to 0.5 Mc Farland units and the inoculum was evenly spread over the Muller Hinton agar plate and was allowed to dry. Antibiotic discs were laid on the plates aseptically with the help of sterile forceps. The plates were incubated anaerobically with gas pack for 24 h at 37°C.

RESULTS

Out of 158 samples analyzed, 34 (21.51%) were found positive for *C. perfringens*. A higher isolation rate was obtained from intestinal tissues (23/34, 67.65%) compared to rectal swabs (11/34, 32.35%). Out of these, 15 (44.12%) samples were isolated from diarrhoeic sheep, 2 (5.88%) were isolated from healthy sheep, 17 (50.0%) were isolated from diarrhoeic lambs. None of the isolates were from healthy lambs, diarrhoeic and healthy goats, diarrhoeic and healthy kids.

All the *Clostridium perfringens* isolates showed characteristic cultural characters viz., increased viscosity of the medium with gas bubbles diffused throughout the medium in thioglycolate broth and black colonies surrounded by zone of opalescence (Fig. 1A and 1B). On Gram's staining, all the positive isolates showed Gram-positive straight rods arranged in singles or in pairs (Fig. 2). All catalase-negative and oxidase-negative Gram-positive rods were further subjected to biochemical tests. A positive reaction for gelatin hydrolysis indicated by liquefaction was observed (Fig. 3). A positive Stormy clot fermentation test observed by formation of shreads of milk clot on the sides of tube with excessive gas production (Fig. 4). All the isolates showed lecithinase activity indicated by the characteristic diffused opalescence around the streak (Fig. 5). Grey, glistening, smooth and round colonies with characteristic double hemolysis (inner zone of complete hemolysis surrounded by outer zone of partial hemolysis) was observed on 5% sheep blood agar (Fig. 6A & 6B) except in few isolates which produced only a single zone of partial hemolysis.

PCR revealed that 34 samples were positive, out of which 30 were found to be type A showing an overall prevalence of 88.23%; Three were found to be type F, showing an overall prevalence of 8.82%; One isolate was found to be type D, showing an overall prevalence of 2.94%.

Sugar fermentation reaction of different toxin types showed different results. *C. perfringens* type A isolated from healthy and diarrhoeic animals that produced alpha toxin exhibited the same pattern of fermentation, i.e., positive for sugars like sucrose, mannose, galactose, maltose, trehalose, raffinose and negative to cellobiose, rhamnose, arabinose and mannitol. Type A isolates with beta2 toxin differ from only alpha toxin-producing type A with galactose, whereas type D ferment all the sugars fermented by type A and also fermented cellobiose. Type F also exhibited similar fermentation pattern with type A except for the sugar raffinose, which exhibited negative reaction. Gas production was observed with the help of Durham's tube. Degree of sugar fermentation among different isolates was represented in Table 3.

Table 3. Degree of sugar fermentation among different toxin types of *C. perfringens*

Sugar	Type A from healthy animal	Type A (α toxin only)	Type A (α, β2 toxins)	Type D (α, ε, entero toxins)	Type F (α, entero toxins)
Sucrose (Su)	+++	+++	+++	+++	+++
Mannose (Mo)	+++	+++	+++	+++	+++
Galactose (Ga)	+++	+++	+	+++	+++

Cont. Table 3.

Table 3., Cont. ...

Sugar	Type A from healthy animal	Type A (α toxin only)	Type A (α, β2 toxins)	Type D (α, ε, entero toxins)	Type F (α, entero toxins)
Maltose (Ma)	+++	+++	+++	+++	+++
Trehalose (Te)	+++	+++	+++	+++	+++
Raffinose (Rf)	++	+	++	+++	-
Rhamnose (Rh)	-	-	-	-	-
Cellobiose (Ce)	-	-	-	++	-
Arabinose (Ar)	-	-	-	-	-
Mannitol (Mn)	-	-	-	-	-

Antibiotic sensitivity test revealed the highest resistance was observed for sulphamethizole (66.67%) followed by co-trimoxazole (58.33%), enrofloxacin (41.67%) and tetracycline (33.33%). The isolates showed highest sensitivity to gentamicin (91.67%), cefotaxime/clavulanic acid (91.67%), norfloxacin (91.67%) followed by chloramphenicol (83.33%), cefotaxime (75.0%) and streptomycin (66.67%) (As per CLSI guidelines - Table 4)

Table 4. Interpretation chart for antimicrobial sensitivity/ resistant patterns of *C. perfringens* isolates – CLSI guidelines

Antimicrobial agent	Symbol	Disc conc. (µg)	Interpretative criteria		
			Resistant (mm or less)	Intermediate (mm)	Sensitive (mm or more)
Gentamicin	GEN	10	12	13-14	15
Cefotaxime	CTX	30	14	15-22	23
Cefotaxime/ Clavilinic acid	CEC	30/10		29-36	
Enrofloxacin	EX	5		22-30	
Chloramphenicol	C	30	12	13-17	18
Tetracyclin	TE	30	11	12-14	15
Co-trimoxazole	COT	25	10	11-15	16
Norfloxacin	NX	10	12	13-16	17
Streptomycin	S	25		17-25	
Sulphamethizole	SM	300		24-34	

DISCUSSION

Clostridium perfringens is a ubiquitous spore-forming anaerobic pathogen that is commonly associated with enteric disease in small ruminants. The overall prevalence of *C. perfringens* observed in this study was 21.51%, which is comparable with the prevalence reported by Hadimli et al. (2012) in lambs. Contrary to this study, a lower prevalence of 9.09% was reported by Soren et al. (2019) and higher prevalence rates of 61.9% and 46.1% reported by Rasool et al. (2017), and Mohiuddin et al. (2020), respectively, in small ruminants.

This study observed 44.12 and 5.88% prevalence rate, respectively of *C. perfringens* in diarrheic and healthy sheep. Contrary, a low prevalence rate of 15.05% and 20.83% was observed by Alsaab et al. (2021) and Ashgan et al. (2013), respectively and a high prevalence of 59.0% and 61.07% was reported by Elsify et al. (2016) and Hayati and Tahamtan (2021), respectively. Several earlier reports (Elsify et al., 2016; Nazki et al., 2017; Hayati & Tahamtan, 2021) also quoted higher prevalence rate for *C. perfringens* in healthy sheep than the results reported in current study in different geographical areas. High prevalence rate of 50% among diarrheic lambs observed in this study was in agreement with Kumar et al. (2014), whereas, Ashgan et al. (2013), Fayez et al. (2013), Hadimli et al. (2012), Rahaman et al. (2013) reported a low prevalence rate of 13.64%, 16.6%, 21.33% and 25%, respectively. The wide variations in prevalence rates reported in previously published research reports may be due to geographical region variations or epidemiological predisposing factors that could affect farm animals.

All the positive isolates exhibited characteristic black colonies surrounded by a zone of opalescence on tryptose sulphite cycloserine (TSC) agar supplemented with egg yolk emulsion. This was in accordance with previous reports of Kalender et al. (2005),

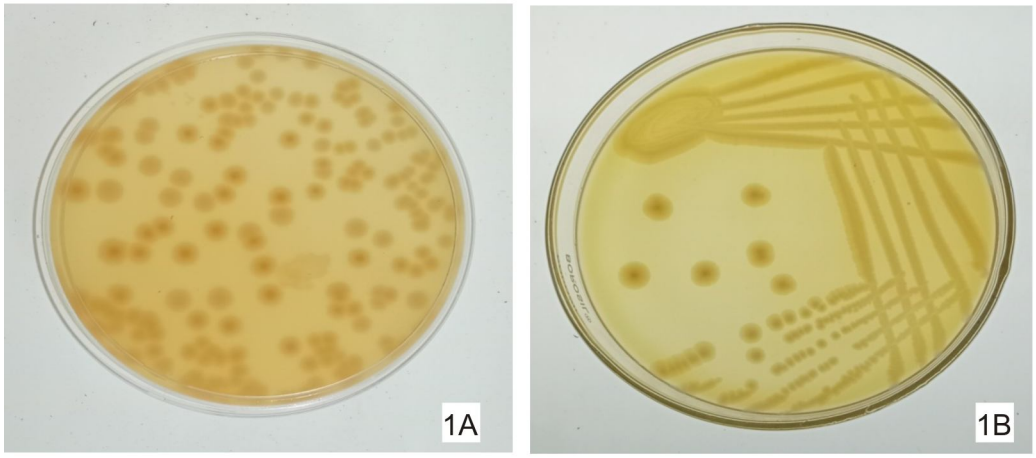


Fig. 1. (1A and 1B) Black colonies surrounded by zone of opalescence on T. S. C agar supplemented with egg yolk emulsion

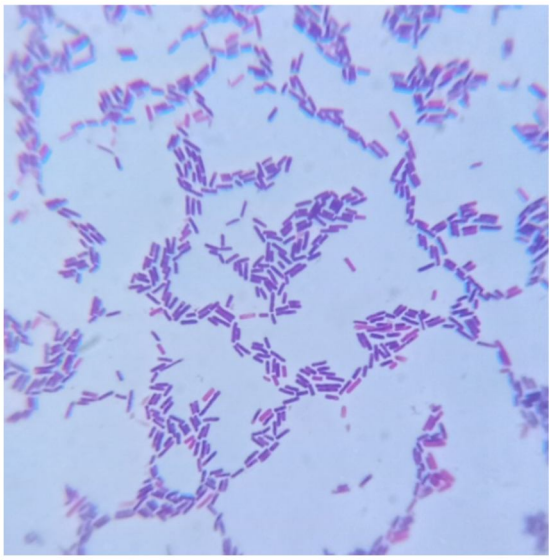


Fig. 2. Gram's staining image of *C. perfringens* (100X)

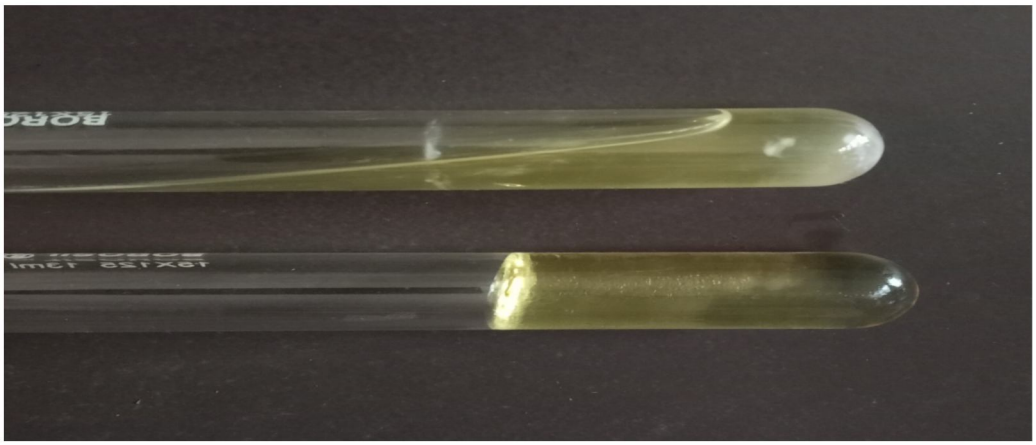


Fig. 3. Gelatin hydrolysis test, *C. perfringens* positive; *E. coli* negative control

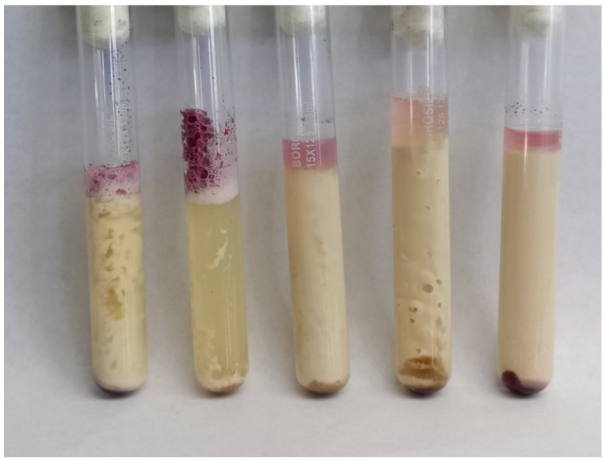


Fig. 4. Stormy clot fermentation test milk clot with gas production

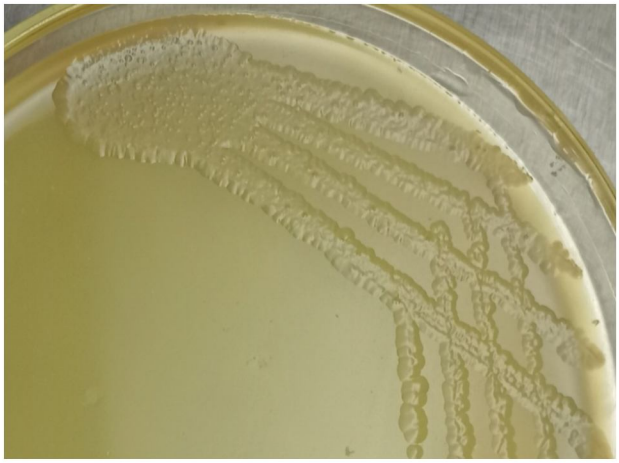
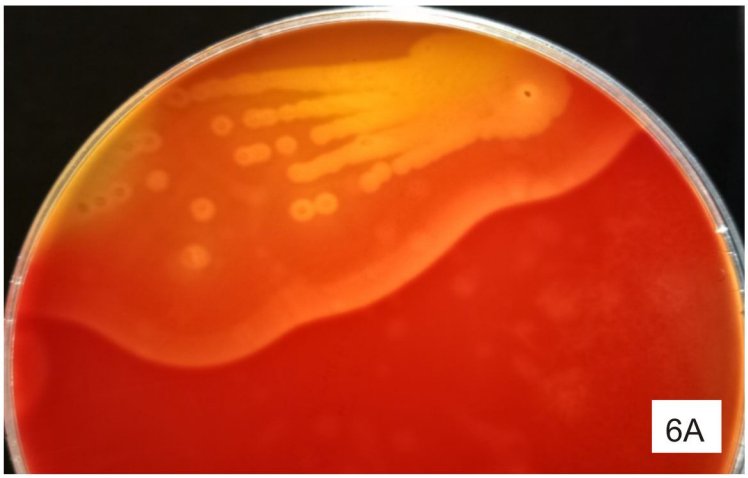


Fig. 5. Lecithinase test -characteristic diffused opalescence around the streak



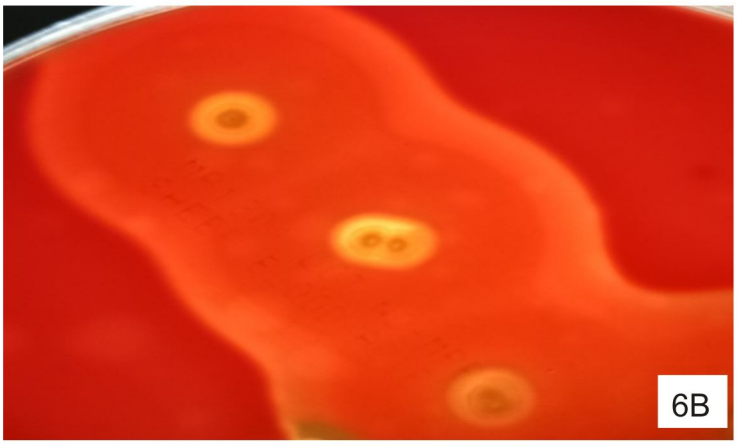


Fig. 6. (6A & 6B) Hemolysis pattern on 5% sheep blood agar characteristic double zone of hemolysis

Elsify et al. (2016), Mohiuddin et al. (2020), Hayati and Tahaman (2021) and Hussain et al. (2022). Most of the isolates produced characteristic grey, glistening, smooth and round colonies on 5% sheep blood agar, which was supported by Hood et al. (1990), Rasool et al. (2017) and Hussain et al. (2022). Difficulties while culturing were faced due to overgrowth of other anaerobic or aerobic organisms along with *C. perfringens*. This can be overcome to some extent by boiling of samples before inoculation into broth.

The staining and biochemical characterization of *C. perfringens* viz. Gram-positive rods and negative for catalase and oxidase reactions, positive reaction for gelatine hydrolysis, stormy clot fermentation test, lecithinase activity were in accordance with the results of many other workers (Hatheway, 1990; Quinn et al., 2002; Agarwal et al., 2009; Ahsani et al., 2011; Rahaman et al., 2013; Alsaab et al., 2021).

In this study, most of the isolates produced characteristic double zone of hemolysis with inner zone of complete hemolysis due to activity of perfringolysin surrounded by outer zone of partial hemolysis due to activity of alpha toxin which is in accordance with reports of Hatheway (1990), Fayez et al. (2013), and Rasool et al. (2017) whereas, few isolates produced only a single zone of partial hemolysis, this may be due to absence of perfringolysin gene in some strains (Uzal et al., 2022).

The prevalence of toxin type A was recorded as 88.24%, type D was 5.88%, and type F was 5.88% among diarrhoeic lambs. The results were in accordance with Kalender et al. (2005) who reported that the prevalence of toxin type A was 95% among healthy sheep, and Kashoo et al. (2018), Omer et al. (2020) also reported that the prevalence of *C. perfringens* type A was 70.65% and 80.8%, respectively. Hadimli et al. (2012) reported 9.33% prevalence of toxin type D from diarrheic calves and Yadav et al. (2017) reported the prevalence of Type F was 5.3% among human diarrhoea cases. Toxin types B, C and E were not detected in anyone of the isolates which was supported by previous studies of Nazki et al. (2017), who reported the absence of *C. perfringens* type B, C and E. Similarly, Kashoo et al. (2018), and Alsaab et al. (2021) also reported that *C. perfringens* type B and type C were absent in sheep samples. Greco et al. (2005), Hadimli et al. (2012) and Elsify et al. (2016) too reported that *C. perfringens* type E was very rare in lambs and kids.

The present study results revealed, *C. perfringens* type A was predominant among all the toxin types in both sheep and lambs in this geographic region. These results were in accordance with many previous studies who reported that *C. perfringens* type A as being the most ubiquitous and prevailing toxinotype (Moussa & Hessan, 2011; Ashgan et al., 2013; Kumar et al., 2014; Elsify et al., 2016; Yadav et al., 2016; Hussain et al., 2017; Rasool et al., 2017; Omer et al., 2020).

All the isolates in this study fermented sugars like sucrose, mannose, maltose, and trehalose with acid and gas production and were negative for rhamnose, arabinose and mannitol. These results go hand in hand with that recorded by Nakashio et al. (1982), Ahsani et al. (2011), Achakzai et al. (2020) and Mohiuddin et al. (2020), whereas, three sugars behaved differently with different isolates. Galactose showed strong fermentation reaction with all the types except type A with beta2 toxin which showed weak fermentation reaction. Variations were also observed in the case of cellobiose sugar, i.e., none of the isolates fermented this sugar except toxin type D. This may be useful to differentiate this toxin type from other types. Raffinose was fermented by all toxin types except toxin type F, which may be sufficient to differentiate it from other toxin types. Hence, these three sugars are useful to differentiate these three toxin types.

The antibiogram results with ten commonly used antibiotics revealed that *C. perfringens* isolates exhibited the highest resistance against sulphamethizole, co-trimoxazole, enrofloxacin and tetracycline. Consistent with these results Achakzai et al. (2020) and Mohiuddin et al. (2020) observed resistance of *C. perfringens* against sulfonamides and tetracycline, respectively. Contrary to these Agarwal et al. (2009) reported *C. perfringens* isolates were sensitive to enrofloxacin gentamicin, cefotaxime/clavulinic acid, norfloxacin, chloramphenicol, cefotaxime and streptomycin. The present results were consistent with the findings of Achakzai et al. (2020) who reported that the isolates showed sensitivity to chloramphenicol. Multidrug resistance was observed in three isolates which exhibited resistance against enrofloxacin, tetracycline, streptomycin, sulphamethizole and co-trimoxazole. Yadav et al. (2016, 2017) also supported the multidrug resistance phenomenon in *C. perfringens*.

In the present study, most of the isolates were characterized as *C. perfringens* type A, and the symptoms were in correlation with yellow lamb disease i.e. diarrhoea, bloat, jaundice, abdominal pain, haemoglobinuria, including deaths of the lambs. However, quantitative estimation of alpha toxin is required for further confirmation. Based on the findings of the present study, it may be recommended to include type A anaculture in

the existing enterotoxaemia vaccine containing only type D anaculture to prevent *C. perfringens* infections in sheep and goat particularly in Krishna and West Godavari districts of Andhra Pradesh.

Conflict of interest: The authors declare that there is no conflict of interest.

Author's contribution: CSMD: Involved not only in acquisition of data but also in statistical analysis of data, interpretation of the results and critically revising the manuscript; SM: Conception and design; KAK: Supervision and revising the manuscript.

Data availability statement: Data underlying the article are available in the article and in its online supplementary material

Ethical approval: The study does not involve direct research on animals.

Use of artificial intelligence tools: No artificial tools were used for this study. The authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

ACKNOWLEDGEMENTS

The authors are thankful to Sri Venkateswara Veterinary University, Tirupati, AP, India for providing funding for conducting the research. The study is a part of the post-graduate research work, "Detection and molecular typing of toxins of *Clostridium perfringens* isolates from small ruminants'' and submitted by the first author to SVVU, Tirupathi.

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Received - 07.04.2025, Accepted - 06.08.2026, Published - 13.10.2026 (Online)
Section Editor: Prof. Neeraj Shrivastava, Editorial Board Member