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Isolation and characterization of *Clostridium perfringens* from small ruminants of Odisha

Niranjan Soren, Rajashree Mishra, Kailash Singh Bisht, Iswar Anand Senapati and Prasana Kumar Rath

Abstract
In the present study 209 samples of sheep and goats were collected from different farms and localities in and around Bhubaneswar, Odisha. Out of which 159 samples were collected from goats and 50 samples were collected from sheep. Both intestinal and fecal samples were collected and processed. A total of 19 samples were found positive indicating the prevalence of the *Clostridium perfringens* to be 9.09% in small ruminant population. Thioglycolate broth, Robertson's cooked meat media (RCM), BHI and TPGY media were used in the present study. Morphological, cultural and bio-chemical characters of all the isolates of *C. perfringens* have been studied in detail. All the 19 isolates of *C. perfringens* were subjected to antibiotic sensitivity test and it was found that out of 15 antibiotic discs tested most of the local isolates were highly sensitive to sparfloxacin, erythromycin, enrofloxacin, doxycycline and amoxicillin. Growth characteristics of *Clostridium perfringens* on TPGY and BHI media revealed that BHI gives good growth in short time.

Keywords: *Clostridium perfringens*, small ruminants, isolation, characterization, antibiotic sensitivity

Introduction
Clostridium perfringens is a common inhabitant of gastrointestinal tract of both humans and animals and in their surroundings. *C. perfringens* is causative organism of food poisoning in man and variety of disease conditions of domestic animals like enterotoxaemia in sheep, goats and calves, struck in sheep, pulpy kidney and lamb dysentery in young lambs. It is evident from various reports from different parts of India. Upadhaya *et al*. (1998) reported that digestive system disorders caused deaths in 15.78% animals, in and around Khanapara area of Guwahati, Assam (1981-1997). Also the mortality among kids and adults was 16.27% and 15.25% respectively [1]. Balakrishnan *et al*., (2005) reported that an outbreak of enterotoxaemia had occurred in sheep due to *C. perfringens* in Karur District of Tamil Nadu, during November 2003 [2]. Senthilkumar *et al*., (2006) studied retrospectively and found the prevalence of enterotoxaemia to be 11.18% in Dharmapuri district of Tamil Nadu with significant relationships between age, sex, breed and deworming [3]. Srinivasan *et al*., (2008) studied the mortality in 5 lambs which ingested large amounts of sand in Tamil Nadu, India and reported that the animals died of bacterial toxins from *C. perfringens* [4]. Singh *et al*., (2008) studied the incidences of mortality from birth to one year old of 5243 Jamunapari kids and reported that death due to enterotoxaemia was 6.73 percent [5]. Vijayalakshmi *et al*., (2011) tested 160 faecal samples collected from sheep enterotoxaemia outbreaks for *Clostridium perfringens* type D and 21 were found to be positive (13%) [6]. These studies reveal how widespread the organism is and its capability to cause diseases.
The Clostridia organisms are rod shaped 3µm to 7µm by 0.4µm to 1.2µm in dimensions. They are Gram positive, facultative anaerobes, large spore forming and non-motile [7]. The organisms are found mostly singly and sometime in pairs or in chain form. In the presence of low concentration (below the MIC) of Beta-lactam antibiotics, *C. perfringens* grows as filamentous which is due to inhibition of septum formation as reported by several workers [8, 9]. The present study is to isolate and characterize *C. perfringens* found in local small ruminants. Cultural and biochemical characteristics along with antibiotic sensitivity patterns were being taken into consideration.

Materials and Methods
This study was carried out in the Department of Veterinary Microbiology, College of

Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar and in the Division of Biological Standardization, I.V.R.I., Izatnagar, U.P. A total of 209 samples were collected from various farms, slaughter houses of Odisha (Table No. 1). Out of 209 samples 84 were intestinal contents and 125 were faecal sample. The standard Reference Strain organisms "Classical enterotoxaemia *Clostridium perfringens* Type D sheep strain" were obtained from Division of Biological Standardization, I.V.R.I., Izatnagar, U.P. for the present study. The isolation of *Clostridium perfringens* was done in Thioglycolate broth, Robertson's cooked meat medium and Blood agar for collected samples as per Skriyachan *et al*. (2010) with necessary modifications [10]. The biochemical tests were carried out as per the methods of Buxton and Fraser, (1977) and Cruickshank *et al*. (1980) [11, 12]. The detail results of the biochemical reactions are depicted in table No. 2. Antibiotic sensitivity testing was conducted for test isolates according to Bauer-Kirby diffusion test with little modifications as per the method of Bauer *et al*., (1966) [13]. A total 15 antibiotic discs having fixed potency/unit were procured from (HiMedia, Mumbai). The Trypticase Peptone Glucose Yeast extract (TPGY) medium and BHI broth were used to study the growth curve of the local strains of *Clostridium perfringens.* The media which gives good growth in short time can be used for isolation of DNA.

Table 1: List of Various state livestock breeding farms, slaughter houses and No. of samples from different area of different districts of Odisha.

Sl. No.	Name	Sheep		Goat	
		SI	SF	GI	GF
1.	SLBF, Chiplima, Sambalpur	1	2	1	5
2.	SBF, Deogan	1	2	-	-
3.	GBF, Jaring, Kalahandi	-	-	1	5
4.	GBF, Dadupaju, Kandhamal	-	-	1	5
5.	GBF, Kuarmunda, Sundargarh	-	-	1	5
6.	LBD Farm, Bhubaneswar	1	2	1	5
7.	Keonjhar	5	3	8	10
8.	Mayurbhanj	4	3	8	10
9.	Khurdha	2	3	7	9
10.	Ganjam	3	2	7	10
11.	Koraput	3	3	8	10
12.	Balasore	2	2	7	10
13.	Jajpur	3	3	6	10
14.	Meat Market, Unit-4, Bhubaneswar	-	-	1	2
15.	Meat Market, CRPF Square, Bhubaneswar	-	-	1	2
16.	Meat Market, Kalpana Square, Bhubaneswar	-	-	1	2
Total		**25**	**25**	**59**	**100**

* SLBF- State Livestock Breeding Farm, SBF- Sheep Breeding Farm, GBF- Goat Breeding Farm, SI- Sheep Intestine, GI- Goat Intestine, SF- Sheep Fecal, GF- Goat Fecal

Table 2: Biochemical characterisation of isolates of *C. perfringens*

Name of biochemical tests	Types of *C. perfringens* isolates.	
	Faecal isolates (n=10)	Intestinal isolates (n=9)
Nitrate reduction	+	+
H_2S production	+	+
Voges Proskauer	-	-
Indole tests	-	-
Catalase	-	-
Methyl red	-	-
Motility	+	+

Results and Discussion

For the isolation of *Clostridium perfringens* all the above samples were processed by a series of enrichment and selective media as described in the materials and methods. 19 samples were found positive for *C. perfringens* on the basis of morphological, cultural and biochemical tests. Present study is in agreement with the findings of previously mentioned investigators [1, 3, 5, 6].

The isolates were identified as *Clostridium perfringens* and compared with the standard "Classical enterotoxaemia *Clostridium perfringens* Type D sheep strain". The organisms were found Gram positive slender rods with square end, arranged single or in chains. Spores were not found when smears were stained from actively growing cultures. These features are typical of *C. perfringens* species and are widely reported in the literatures [8, 9, 10, 14]. The biochemical results of present study were positive for nitrate reduction, H_2S production and motility. Positive isolates were negative for catalase, methyl red, Voges Proskaur and indole test. Similar type of biochemical reactions were reported by various investigators [10, 15].

The Antibiotic sensitivity tests of *C. perfringens* isolates were performed using 15 different antibiotic discs. The results show that Amoxicillin, Enrofloxacin, Erythromycin, Doxycycline and Sparfloxacin were sensitive for the local *C. perfringens* isolates. Sparfloxacin showed the maximum zone of inhibition followed by erythromycin. Chloramphenicol, Gentamycin, Nalidixic acid, Streptomycin, Tetracycline, Levofloxacin, Neomycin, Oxytetracycline, Kanamycin and Penicillin-G were found resistant. The results of susceptibility of *C. perfringens* field isolates are given in the Table No. 3. Sensitive strain for these antibiotics has also been reported earlier [16, 17]. In general Clostridial species have been found to be sensitive for penicillin but the present isolated strain has been found resistant which is in support of the findings [18, 19]. Whereas penicillin susceptibility was also reported [20, 17]. This indicates that the resistance pattern for penicillin is variable.

Tetracycline resistance strain of *C. perfringens* has been reported in the literatures [18, 20]. Hence the resistance to tetracycline is in tune with the finding of the present study. But sensitive strain for tetracycline is frequently encountered and it has been found recently [17].

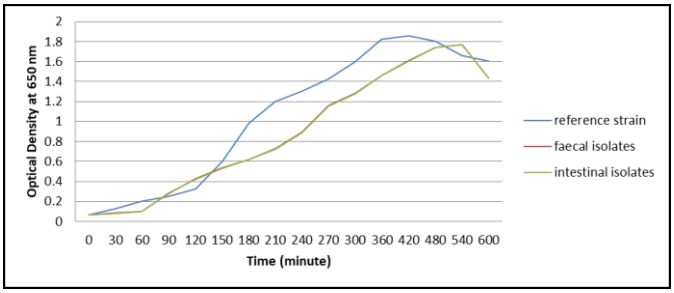
The isolates gave better growth over BHI broth in comparison to that over TPGY Media. In case of TPGY Media the visible

growth of reference Classical enterotoxaemia *C. perfringens* Type D sheep strain started after 1 hour of incubation which gradually increased up to 7 hours of incubation at 37°C with O.D. of 1.855, growth remained in stationary phase up to about 10 hours of incubation after which decline phase started. The visible growth of test *C. perfringens* strain was seen after 1 hour of incubation and reached to its maximum at 9 hours of incubation with O.D. of 1.768. The stationary phase remained up to 10 hours of incubation after which growth started receding. The detail of the growth has been shown in Graph 1.

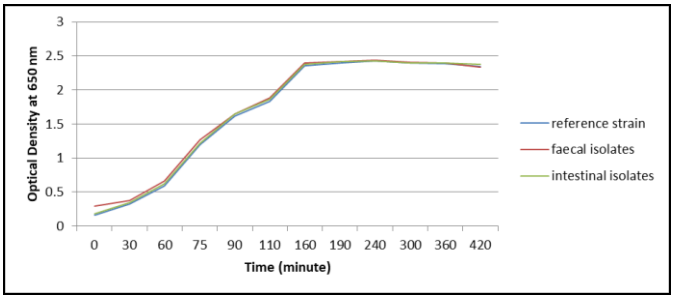
In case of Brain Heart Infusion (BHI) broth the visible growth of reference strain started after 1 hour of incubation which reached its peak at 4 hours of incubation with maximum O.D. of 2.43. The stationary phase remained up to 10 hours of incubation after which growth started receding. The visible growth of test *Clostridium perfringens* strain was seen after 1 hour of incubation. Maximum growth was observed at 4 hours of incubation with O.D. of 2.44. It remained in stationary phase up to 10 hours of incubation, after which decline phase started. The detail of growth has been shown in Graph 2. The BHI broth has been found to be better medium as it supported maximum growth (highest OD) as obtained along with better plasmid yield. As there was supplementation of glucose in BHI broth medium the increase of O.D. was much faster than that of TPGY medium. Similar result has been reported [21, 22]. They all reported that the combination of peptone, glucose and yeast extract give better growth of *C. perfringens*.

Table 3: Antibiotic susceptibility of local *C. perfringens* isolates.

Sl. No.	Antibiotic	Disc potency (µg/ IU)	Zone of inhibition (mm)		Interpretation	
			Faecal isolates (n=10)	Intestinal isolates (n=9)	Faecal isolates (n=10)	Intestinal isolates (n=9)
1	Amoxicillin	10 µg	20mm	18mm	Sensitive	Sensitive
2	Chloramphenicol	30 µg	Nil	Nil	Resistant	Resistant
3	Enrofloxacin	10 µg	28mm	25mm	Sensitive	Sensitive
4	Gentamycin	10 µg	Nil	Nil	Resistant	Resistant
5	Nalidixic acid	30 µg	Nil	Nil	Resistant	Resistant
6	Streptomycin	10 µg	Nil	Nil	Resistant	Resistant
7	Tetracycline	30 µg	Nil	Nil	Resistant	Resistant
8	Erythromycin	15 µg	30mm	27mm	Sensitive	Sensitive
9	Doxycycline	30 µg	25mm	23mm	Sensitive	Sensitive
10	Sparfloxacin	5µg	33mm	31mm	Sensitive	Sensitive
11	Levofloxacin	30 µg	Nil	Nil	Resistant	Resistant
12	Neomycin	30 µg	Nil	Nil	-do-	-do-
13	Oxytetracycline	30 µg	Nil	Nil	-do-	-do-
14	Kanamycin	30 µg	Nil	Nil	-do-	-do-
15	Penicillin-G	10 IU	Nil	Nil	-do-	-do-



Graph 1: Growth curve of *C. perfringens* in TPGY Media with Reference strain.



Graph 2: Growth curve of *C. perfringens* in BHI Broth (Difco) supplemented with 1% glucose with Reference strain.

Conclusion

In the present study 209 samples were collected from sheep and goats present in different farms and localities in and around Bhubaneswar, Odisha. A total of 19 samples were found positive indicating the prevalence of isolation is 9.09% in small ruminant population. All the 19 isolates of *C. perfringens* were subjected to antibiotic sensitivity test and it was found that out of 15 antibiotic discs tested most of the local isolates were highly sensitive to sparfloxacin, erythromycin, enrofloxacin, doxycycline, amoxicillin. Both BHI and TPGY media were used in the present study. Almost similar characters of all isolates were observed in both media. BHI showed better growth in comparison to TPGY. So BHI can be used as growth media for better DNA isolation in Clostridial species as it can give luxurious growth in short time.

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