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Isolation of *Clostridium perfringens* from Goats and Sheep of the Khuzdar district of Balochistan, Pakistan

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Key words: *Clostridium perfringens*, Goats, Sheep, Khuzdar, Balochistan

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Abstract

In present study, causal agent of necrotic enteritis (NE) in goats and sheep of Khuzdar district, Balochistan was isolated. Total 200 samples were collected out of that 66.5 % were positive for *Clostridium Perfringens* while 33.5 % were negative. The area wise distribution of positive cases between Khuzdar 8 %, Wadh 18 %, Nall 16 %, Zehri 13.5 % and Mulla was 11%. Tehsil Wadh was highly infected in district. Whereas sheep and goats wise results showed that sheep (40.5 %) was more affected with enterotoxaemia as compared to goats (26 %). Where sex wise ratio among positive cases has been 28.5 % males (sheep 16 % and 12.5 %) and females 38 % (sheep 23 % and goats 15 %). However, age wise distributed was 8 % in one month, 18.5 % in six months, 32 % in one year, and 7.5 % in two years. *C. Perfringens* was confirmed through gram staining using different biochemical tests (IMVIC, Sugar fermentation, Catalase, Oxidase, Gelatin, Litmus milk and Lecithinase) and electron microscopically. Under electron microscope *C. perfringens* was 1.3-1 9.0µm in length and width 0.6-2.4µm. Different drugs result showed that *C. Perfringens* was sensitive to Chloramphenicol, Amoxycillin, Metronidazole, Vancomycin, Ciprofloxacin and Penicillin G while resistant to Polypeptides, Glycopeptides, Aminoglycosides, Cephalosporin's, Lincosamides, Macrolides and Sulfonamides. The animal trial was carried out to check the effect of *C. Perfringens* in different organ. The *C. Perfringens* affected the intestine, liver, kidney, central nerves system and gall bladder of the mice.

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Introduction

Enterotoxaemia caused by *Clostridium perfringens* is a fatal enteric disease that affects all species of animals (Anders, 2006). *C. perfringens* is a gram-positive, anaerobic bacterium and it is widely spread in the environment (Javed *et al.*, 2012). In animal and human being, it is commonly found in the gastrointestinal tract.

In gastrointestinal tract *C. perfringens* are present in the low numbers (Songer, 1996). These organisms produce little toxin and under normal conditions are removed by normal gut movements or are inactivated by enterotoxaemia circulating antibodies but when the intestinal environment is altered by sudden changes in diet or other factors, *C. perfringens* proliferates and produces potent toxins that act locally or absorbed into the general circulation with usually devastating effects on the host (Songer, 1996).

Enterotoxaemia is one of the most frequently occurring diseases of sheep and goats in Khuzdar. In Khuzdar, sheep and goats are estimated at 37.97 and 3.92 million heads, respectively (Pakistan Bureau of statistics, 2006). They are economically the most important farm animals in the region, serving as major sources of meat, milk and income for a large sector of the population (Greco *et al.*, 2005). The health and well-being of the livestock is of supreme importance.

It is firmly believed that optimum production cannot be achieved without protection from the different livestock disease particularly enterotoxaemia (Chandran *et al.*, 2010; Wang *et al.*, 2011).

The outbreaks of these diseases are disasters for the farmers and may put them out of their business by imposing excessive economic losses (Gad *et al.*, 2011). So, it is quite necessary to prevent these infectious diseases to ensure wellbeing and prosperity of people by minimizing, effective treatment to prevent economic losses. Therefore, the present study was design to isolate the C. *perfringens* from sheep and goats of Khuzdar District through different techniques.

Materials and methods

Collection of samples

A total 200 fecal samples were collected from sheep and goats suffering from diarrhoea (Fayez *et al.*, 2013; Ossiprandi *et al.*, 2013). The samples were collected aseptically in polyethylene sachets with UV treated contamination free with double volume of phosphate buffer solution (PBS) and transported to laboratory in cold box for further laboratory process at CASVAB, University of Balochistan, Quetta for microbiological analysis.

Isolation and identification

Fecal samples were inoculated deep into Robertson's cooked meat broth (RCMB). The inoculated RCMB tubes were placed in water bath for a period of 10-15 min at 80°C to eliminate the non-spore forming aerobic bacteria. Finally, the RCMB tubes were incubated in anaerobic jar at 37°C for 24 to 48 h. Colonies with morphology suggestive of *C. perfringens* were further identified by gram staining, biochemical test (IMVIC, sugar fermentation tests, catalase test, oxidase test, stormy milk, gelatine liquefaction test, lecithinase test) and electron microscopically (Miah *et al.*, 2011).

Antibiograms of isolates

Standardized antibiotic sensitivity test was performed on Mueller Hinton agar using disc diffusion Bauer technique and McFarland Turbidity Standard method 0.5 following CLSI protocols. Isolates were considered as sensitive and resistant to a particular antimicrobial agent on the basis of inhibitory zone.

Electron microscopy of the C. perfringens

Scanning electron microscopy was performed to checked the actual size and shape of the isolated genera of the *C. perfringens* bacteria by taking the cultured colony and suspended into BPS (phosphate buffer solution) solution after the centrifugation shaked with the 1.5% glutaraldehyde and allowed for fixation then dehydrate the free cell with acetone. Take the drop on silver tape and allowed drying in hot air. Dried free cell seen under the scanning electron microscope.

Animal trail on mice

To test the pathogenecity of strain, lab animals has been selected and 0.5mL growth suspension having 1x10^9 cfu/mL of each sample was injected intra peritoneally. The post-mortem of the dead animal was performed (Fisher *et al.*, 2006).

Results

Among 200 fecal samples 66.5% has been *Clostridium perfringens* positive and 33.5% was negative as shown in the Fig. 1. It was observed that out of positive samples Khuzdar 8%, Wadh 18%, Nall 16%, Zehri 13.5% and Mulla was 11% positive for enterotoxaemia. Wadh Tehsil was highly infected with enterotoxaemia among five Tehsils of Khuzdar district as shown in the Fig. 2.

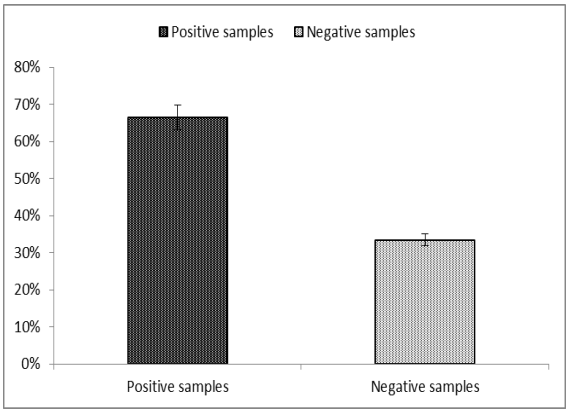

Fig. 1. Positive and negative samples of enterotoxaemia at Khuzdar district.

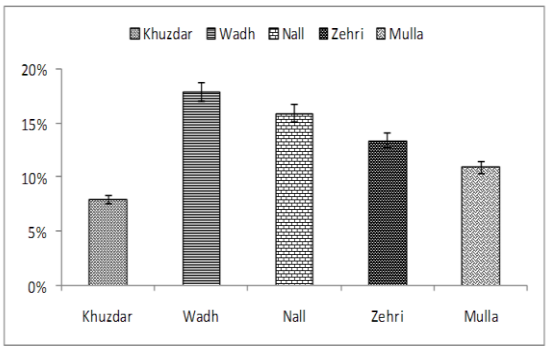

Fig. 2. Enterotoxaemia in five tehsils of Khuzdar district of Balochistan.

Enterotoxaemia in the sheep and goats of Khuzdar

Tehsil wise ratio of *C. perfringens* presence in sheep and goats of Khuzdar was 5%, Wadh 11%, Nall 9.5%, Zehri 8% and Mulla 7%.

The total 40.5% samples of sheep were positive. While tehsil wise presence of *C. perfringens* in goats was Khuzdar 3%, Wadh 7%, Nall 6.5%, Zehri 5.5% and Mulla 4%. The total 26% of goat samples were positive as shown the Fig. 3.

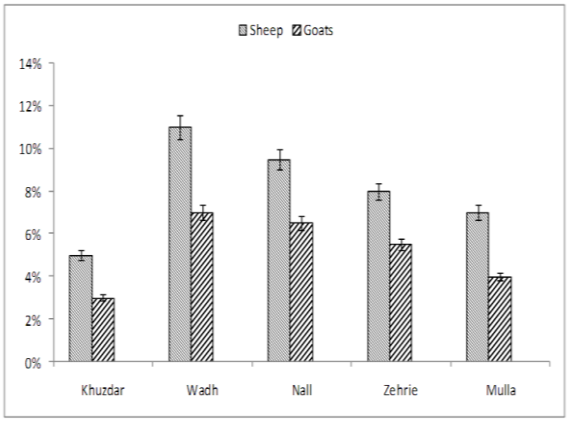

Fig. 3. Enterotoxaemia in the sheep and goats of Khuzdar district.

Age wise distribution of the positive sample of enterotoxaemia

The age wise distributed was 8% in one month, 18.5% in six month, 32 % in one year, and 7.5% in two year in sheep and goats. In sheep 5.5% in one month, 7% in six month, 19% in one year and 4% in two years while in goats 3%, 11.5%, 13% and 3.5% respectively as shown in the Fig. 4.

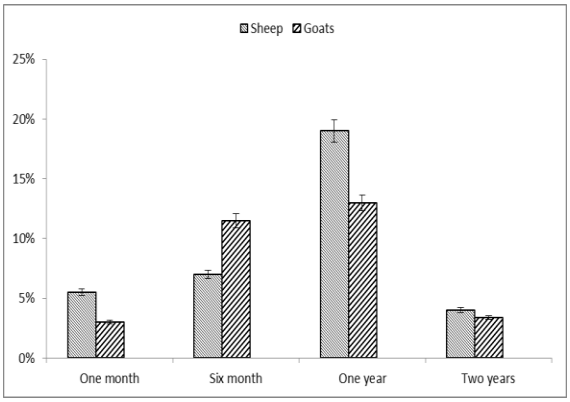

Fig. 4. Distribution of the positive samples in sheep and goats of district Khuzdar.

Comparison of enteroxaemia between male and female of enterotoxaemia in sheep and goats

The males were 28.5 % (sheep 16 % and goats 12.5 %) and females were 38 % (sheep 23 % and goats 15 %) positive for *enterotoxaemia* as shown in the Fig. 5.

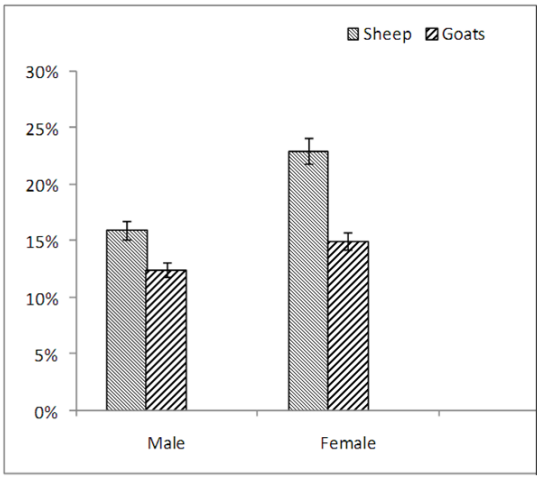

Fig. 5. Comparison of enterotoxaemia between male and female in district Khuzdar.

Conformation through biochemical tests

The present study was conducted to identify the causal agent of necrotic enteritis, which has been effecting goats and sheep population in Khuzdar District of Balochistan and causing heavy economic losses.

Routine methods of bacterial cultures in different media, specific colony characters, microscopic examination, staining techniques, electron microscopy and different types of biochemical tests (IMVIC and sugar fermentation, catalase, oxidase, gelatine liquefaction, stormy milk and lecithinase) were used for the isolation and identification of *C. perfringens* as shown in the Table 1.

Table 1. Different biochemical test and sugar fermentation tests for identification *C. perfringens*.

S.No	Biochemical test properties		*Clostridium perfringens*
1	Gram staining		Gram positive
2	Shape		Rods
3	IMVIC	Indole test	-
		Methyl red test	+
		Voges-Proskauer test	-
		Citrate test	-
4	Litmus/Stormy milk test		+
5	Catalase test		-
6	Oxidase test		-
7	Urease test		-
8	Gelatine liquefaction test		+
9	Lecithinase test		+
10	Sugar fermentation	Fructose	+
		Maltose	+
		Mannitol	-
		Dulcitol	-

Electron microscopy of the C. perfringens

The positive samples were examined under the electron microscope for their shape and size. The *C. perfringens* was rod shaped and length 1.3-1 9.0 µm and width 0.6-2.4 µm as shown in the Fig. 6.

Antibiogram sensitivity test

The *C. Perfringens was* sensitive to chloramphenicol (chloramphenicol 27 mm), pencillin (amoxycillin 27 mm, pencilling G 9 mm), glycopeptides (vancomycin 16 mm), quinolones (ciprofloxacin 14 mm) and metronidazole (metronidazole 17 mm). While multidrug resistance pattern was seen with Polypeptides (colistine sulphate, polyxin B), aminoglycosides (amikacin, kanamycin, gentamycin, streptomycin), cephalosporin's (cefotaxine sodium), Lincosamides (lincosamycin), macrolides (erythromycin) and sulphonamides (trimethoprim) as shown in Table 2.

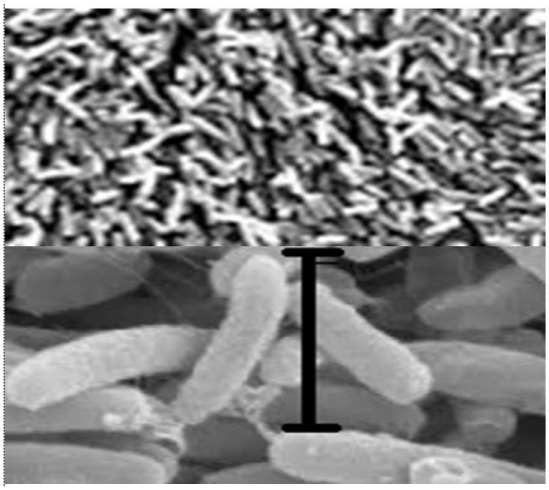

Fig. 6. Electron micrograph of *C. perfringens*.

Table 2. Antibiotic resistance and sensitivity test against *C. perfringens*.

Class	Antibiotics	Abbreviation	µgs	Zone (mm)
Chloramphenicol	Chloramphenicol	C	30	27 mm
Penicillin	Amoxycillin	AML	10	27 mm
	Penicillin G	P	10	9 mm
Polypeptides	Colistine Sulphate	CT	30	00
	Polymyxin B	POL	30	00
Glycopeptides	Oxolinic acid	OXA	10	00
	Vancomycin	VA	30	16 mm
Quinolones	Ciprofloxacin	CIP	5	14 mm
Aminoglycosides	Amikacin	AK	30	00
	Kanamycin	K	30	00
	Gentamycin	CN	10	00
	Neomycin sulphate	K	30	00
	Streptomycin	STR	10	00
Flagyl	Metronidazole	MTZ	25	17 mm
Cephalosporin's	Cefotaxine Sodium	CTX	30	00
Lincosamides	Lincomycin	L	30	00
Macrolides	Erythromycin	E	15	00
Sulfonamides	Trimethoprim	W	5	00

Animal trial on mice

The pure isolated pathogen bacteria were inoculated in the mice with direct internal peritoneal injection and one had placed control. After inoculation of pathogens the animal was observed every 6 hours. The sign such as diarrhoea, anorexia, bally kicking due to colic and muscle tremors were observed. While on autopsy the body, weight was reduced, internal organ like pulpy kidney, shrunk liver and necrotic enteritis were observed as shown in Fig. 7.

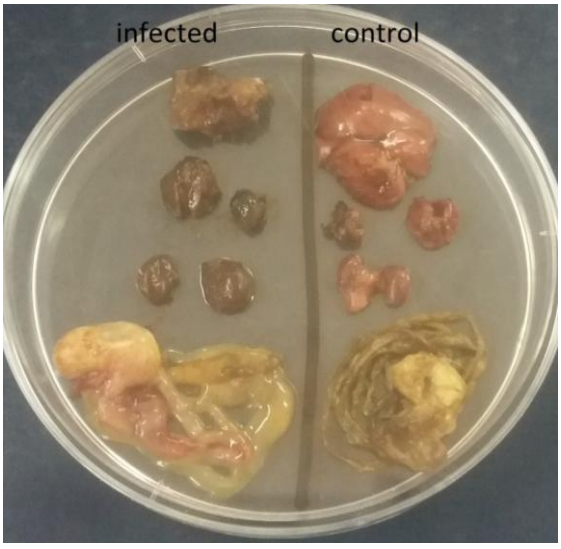

Fig. 7. Mice trial against *C. perfringens* bacteria.

Discussion

Total 200 samples were collected out of that 66.5% showed *Clostridium perfringens positive* while 33.5% were negative.

The area wise distribution was Khuzdar 8%, Wadh 18%, Nall 16%, Zehri 13.5% and Mulla 11% positive for *C. perfringens*. Sheep had 40.5% and goats 26 % positive cases of *C. perfringens* while 28.5% male (Sheep 16% and Goats 12.5 %) and 38% female (Sheep 23 % and Goats 15%) positive for *C. perfringens*. The age wise distribution was 8% in one month, 18.5% in six months, 32% in one year, and 7.5% in two years.

The present study showed that the sheep were more infected as compared to the goats. The age and sex comparison revealed that sheep were highly infected then goat species. Our finding was same as reported by Abildgaard *et al.*, (2009). All the morphological characteristics and biochemical tests were same as described by (Phukan *et al.*, 1997). The size of C. perfringens was 1.3-1.9 µm in length 0.6-2.4 µm in width while our result was same as descried by (Das and Adarsh (2012).

Different classes of antibiotic showed different result. The classes Chloramphenicol, Penicillin, Metronidazole, Quinolones, Glycopeptides, Cephalosporin's classes showed sensitivity for *C. perfringens* bacteria. While Glycopeptides, Polypeptides, Aminoglycosides, Cephalosporin, Lincosamides, Quinolones, Macrolides and Sulphonamides were resistant our findings were same as described by Osman and Elhariri (2013).

The signs such as diarrhoea, anorexia, bally kicking due to colic and muscle tremors were observed. While autopsy revealed reduced body weight, internal organ like pulpy kidney (swollen kidney tissue due to rapid degenerate), liver shrunk and necrotic enteritis were recorded our findings were same as described by Hafez (2010); Francisco *et al.* (2009).

Conclusion

During the collection of the samples. It was observed that the farmers have lacking knowledge and unawareness about enterotoxaemia and enterotoxaemia vaccine. The seasonal vaccination is the solution to control the disease spread in the rainy seasons from month of March to August.

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