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ISOLATION AND IDENTIFICATION OF *CLOSTRIDIUM PERFRINGENS* CAUSING ENTEROTOXAEMIA IN BOVINE OF KACCHI DISTRICT BALOCHISTAN.

Running Title.

***Clostridium perfringens* causing enterotoxaemia in bovine of Kacchi district Balochistan.**

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Abstract:

The causal agent of necrotic enteritis was isolated from the bovine of kacchi district Balochistan. Total 200 fecal samples were collected, out of which 74% samples were positive and 26% were negative for Clostridium perfringens. The age wise distribution was 20 % in 6 month, 16 % in 1 year, 13% in 2 years, 11% in 3 years, 8% in 4 years and 6% in 5 years. However species wise results showed that the cattle were 54% and buffaloes were 20% affected with enterotoxaemia. The season wise result showed that the incidence of enterotoxaemia was more in spring season. Clostridium perfringens was confirmed through different biochemical tests and gram staining. Antibiograms result showed that Clostridium perfringens was sensitive against Chloramphenicol, Penicillin, Tetracycline, Glycopeptides, Quinolones, Lincosamides and Macrolides classes while resistance to Polypeptides, Flagyl and Sulfonamides classes. The animal trail showed that Clostridium perfringens affected the vital organ. The hemorrhagic lesion on lungs, kidneys, and intestine were observed at autopsy. Whereas confirmed by PCR showed clear bands of 233bp of CPE gene.

Key words: *Clostridium Perfringens, Bovine, Kacchi, Balochistan, PCR.*

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Please cite this article in press ***Muhammad Kamran Taj*** *et al.,* ***Isolation and Identification of Clostridium Perfringens Causing Enterotoxaemia in Bovine of Kacchi District Balochistan,*** *Indo Am. J. P. Sci, 2018; 05(04).*

INTRODUCTION:

Clostridium perfringens are spore forming, rod shape, gram positive and anaerobic bacteria. *Clostridium perfringens* causes enterotoxaemia in human being and animals. They are found in natural environment, particularly in soil and water as well as in decomposing animal and plant matters [1].

Clostridium perfringens is a harmless member of the normal micro flora, but under certain conditions, it can multiply rapidly and degradative enzymes that are associated with serious enteric disease [2]. *Clostridium perfringens* is classified into five types; A, B, C, D and E, based on the synthesis of four major lethal toxins, alpha, beta, epsilon and iota [3]. Alpha toxin is produced by almost all strains of this bacterium [4] and proposed to play a major role in both histotoxic infections as gas gangrene, and enteric infections as human food poisoning [5]. The toxin has also been proposed to play an important role in several diseases of animals including enterotoxaemia in calves [6].

In Bovine enterotoxaemia is a major cause of mortality in veal calves. The enterotoxaemia in bovine causes high fertility rate, hemorrhagic lesion in the small intestine, sudden death in absences of clinical signs [7]. The enterotoxaemia accounts for approximately 20% of the mortalities in calves, compared to 4% in dairy and mixed breed veal calves [8].

In Kacchi district, cattle and buffalos are estimated at 151736 and 4151 million herds respectively [9]. They are economically important for farmer and large income source for population [10]. The outbreak of enterotoxaemia is disasters for the farmers and may put them out of their business by improving excessive economic losses, so it is quite necessary to prevent enterotoxaemia to ensure wellbeing and prosperity of people by minimizing effective treatment to prevent economic losses. Therefore, the present study was design to isolate the *Clostridium perfringens* from bovine of Kacchi district through different techniques.

MATERIALS AND METHODS:

Collection of samples:

A total of 200 fecal samples were collected from cattle's and buffalos of kacchi district. Samples were collected in tube and brought to the Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB) for microbiological examination.

Isolation and identification:

Fecal samples were inoculated into the Reinforced Clostridial Medium (RCM). The RCM inoculated tubes were placed in the water bath for 15 to 20 minutes at 80 ˚C to eliminate the non-spore forming bacteria. The RCM inoculated tubes were incubated in an aerobic jar for 24 to 48 hours at 37 ˚C. For further confirmation colonies and morphology of *Clostridium perfringens* were identified through Gram staining, different biochemical tests (IMVIC, Oxidase test, Gelatin liquefaction test), sugar fermentation tests (Glucose, Lactose, Maltose, Sorbitol, Dextrose and Mannitol) and PCR.

Antibiograms of *Clostridium perfringens*:

Mueller Hinton agar (MHA) were used for antibiotic sensitivity, by disc diffusion method following CLSI protocols. On the bases of inhibitory zone the isolated were consider sensitive and resistance [7].

Lab Animals trail to check the effect of *Clostridium perfringens*:

Mice were selected to test the pathogenicity of *Clostridium perfringens* on different organs**.** The $1X10^9$ growth suspension of 0.5 ml was injected intraperitoneally. The sign were observed after 6 hrs Interval. The postmortem was done on the dead animal to identify the lesion on different organs.

Molecular detection:

Polymerase chain reaction was used for colonies which were identifies as *Clostridium perfringens*. Entire genomic DNA was extracted from samples using genomic DNA purification kit (Promega, USA)[11]. In this study primers of the following arrangement F (GGAGATGGTTGGATATTAGG), R (GGACCAGCAGTTGTAGATA) were practiced that amplified a 233-bp of portion of CPE gene of identified isolate. PCR was performed in 25µl mixture containing 9µl grade water, 1µl each set of primer (forward and reverse), 12µl master mix (2x AmpMasterTM Tag) and 2µl of DNA template. Samples were subjected to the following thermocycling process in 94°C for 5 min followed by 25 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, final extension at 72°C for 10 min. PCR product was run on agarose gel (1.5%) and bands were visualized under UV light.

RESULTS:

Total 200 fecal samples were collected from Kacchi district, out of which 74% has been positive for *Clostridium perfringens* while 26% were negative as

shown in Fig-1

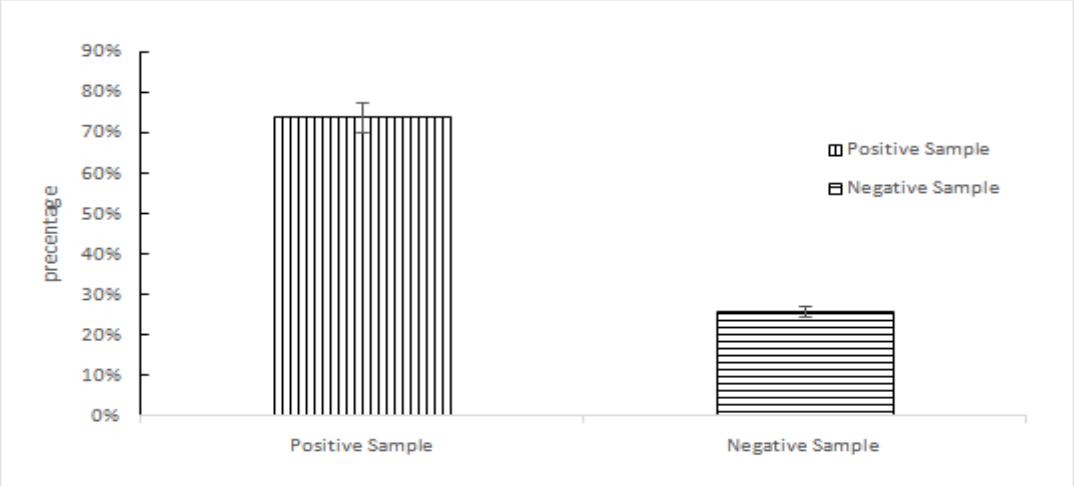

Fig-1: Positive and negative samples of *Clostridium perfringens* isolated from fecal sample of kacchi district, Balochistan.

The age wise enterotoxaemia distribution was 20 % in 6 month, 16 % in 1 year, 13% in 2 years, 11% in 3years, 8% in 4 years and 6% in 5 years as shown in Fig-2.

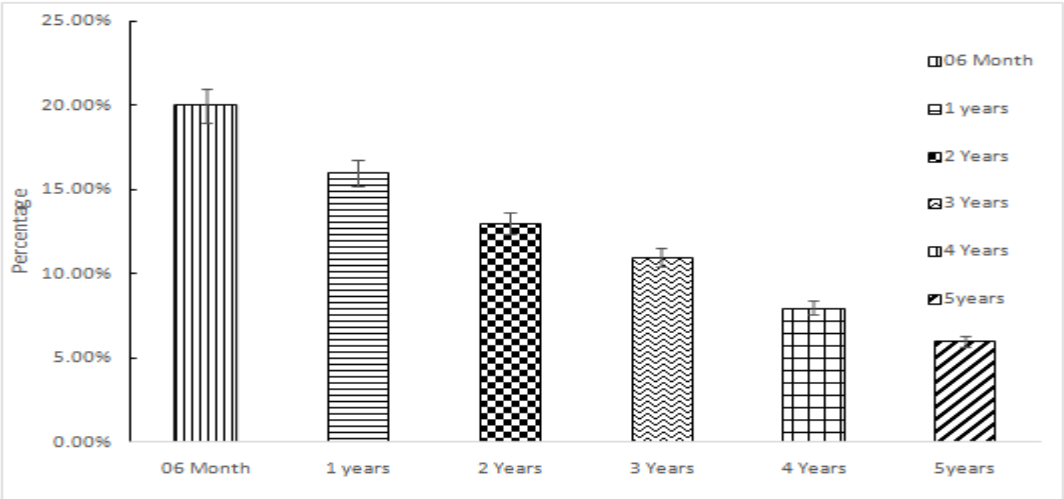

Fig- 2: The age wise ratio showed that bovine from 6 months to 1 year of age were more effected with enterotoxaemia.

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The cattle of kacchi district were 54% and buffaloes were 20% affected to enterotoxaemia. The buffaloes of kacchi district were less affected then cattle as shown in Fig-3.

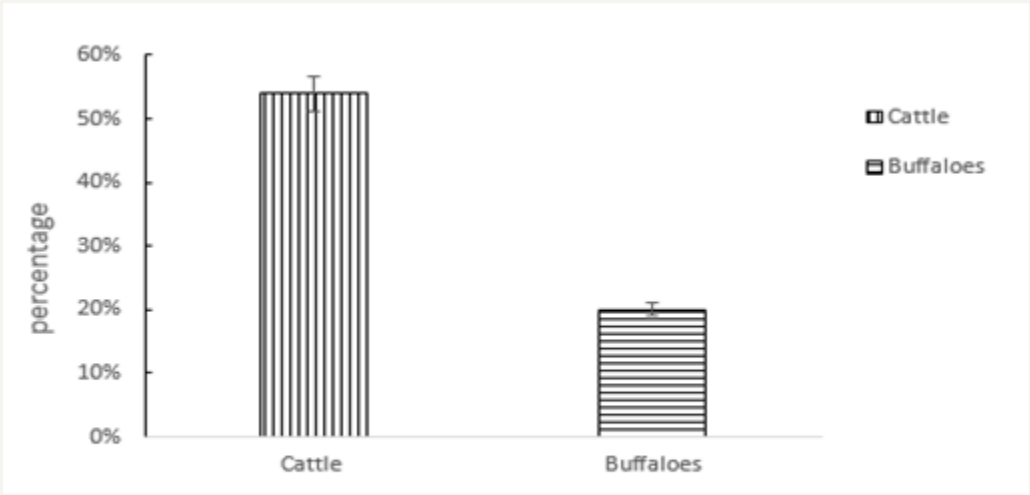

Fig-3: The cattle were more affected as compare to buffaloes of district

The season wise result showed that the incidence of enterotoxaemia was more in spring season (28%) and autum (20%) as compared to other season (summer 15% and winter 11%) as shown in Fig-4.

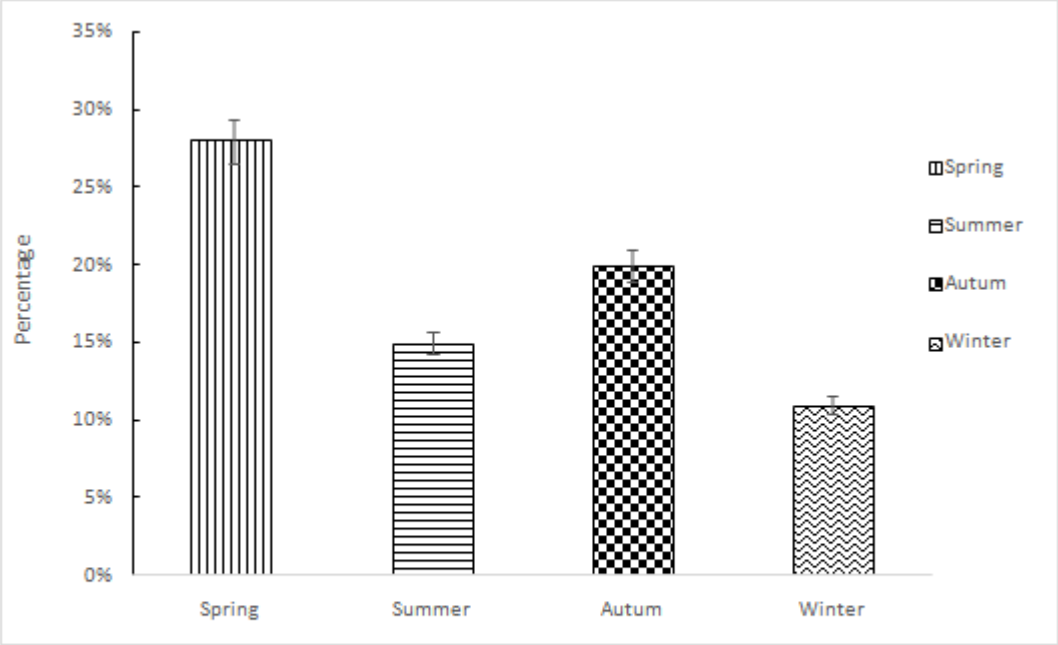

Fig -4: In spring and autum the disease incidence were more as compared to others seasons.

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The pure *Clostridium perfringens* were inoculated in the mice with direct internal peritoneal injection and one had control. After inoculation of pathogens the animal were observed every 6 hour .The sign and symptoms such as kicking belly due to colic pain , diarrhea, anorexia and muscle tremors were observed. While on postmortem the body weight was reduced, necrotic enteritis, hemorrhages in vital organs like pulpy kidney and liver shrunk, abomastitis lesion and necrotic hemorrhage lesion in jejunum was observed as shown in Fig-5.

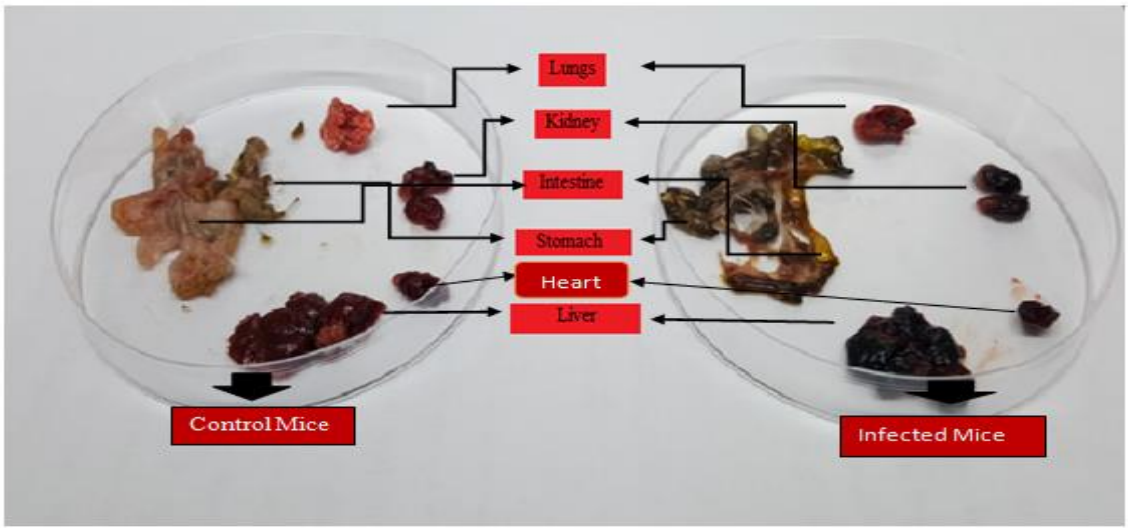

Figure-5: *Clostridium perfringens* affect on mice

In the current study, molecular diagnosistic procedure based on gene specific polymerase chain reaction assay was practice to detect *Clostridium perfringens.* All the isolated of *Clostridium perfringens* used in current study produced the predicted size of 233-bp amplicon CPE gene as shown in Fig-6

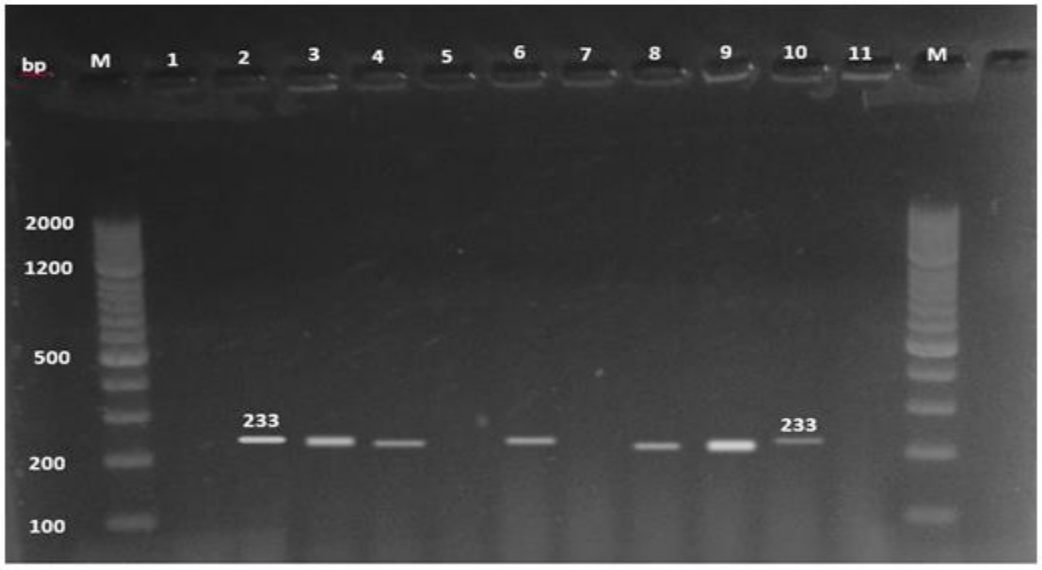

Figure-6: :Molecular identification of Clostridium *perfringens* in Cattle & Buffaloes samples by direct using CPE gene specific primers. Lane M: 100 bp plus DNA ladder. Lane 1 and 11 negative control. Lane 2 to 10 positive samples

Confirmation through biochemical tests

The present study was conducted to identify the causal agent of necrotic enteritis, which has been effecting cattle and buffalo's population in kacchi district of Balochistan and causing heavy economic losses. Routine methods of bacterial culture in different media, specific colony characters, microscopic examination, staining techniques, biochemical tests (IMVIC, catalase, oxidase, gelatin liquefaction, stormy milk and lacithhinase) and sugar fermentation tests were used for identification of *Clostridium perfringens* as shown in Table-1.

Table-1: The biochemical test of *Clostridium perfringens* isolation from bovine fecal samples.

Biochemical test of *Clostridium perfringens*		
Gram Staining	Crystal violet color	
Shape	Rod, Rectangular, Endospore (Sub terminal, Central)	
IMVIC	Indole	Negative
	Methyl red	Negative
	Voges-prokaures	Negative
	Catalase	Negative
Others	Urease test	Negative
	Gelatin liquefaction test	Positive
	Oxidase test	Negative
	Casine test	Positive
	Litmus milk test	Positive
	Motility test	Negative
	H_2S gas production test	Positive
	Starch test	Positive
	Lacithhinase test	Positive
Sugar Fermentation Test	Glucose	Positive
	Lactose	Positive
	Maltose	Positive
	Sorbitol	Positive
	Dextrose	Positive
	Mannitol	Negative
Selective RCM Media	Opaque white color, smooth, regular convex colonies appear	

Drugs sensitivity test

The *Clostridium perfringens* was sensitive to Chloramphenicol (Chloramphenicol 25mm), (Amoxicillin 27mm) Glycopeptides (Vancomycin 22mm), Quinolones (Ciprofloxacin 30mm) (Kanamycine 19mm), Tetracycline (Tetracycline 27mm), Lincosamides (Lincosamycin 20mm), Macrolides (Erythromycin 22mm) classes while resistances to Flagyl (Metronidazole), Sulfonamides (Trimethoprim) and Polypeptides (Colistin suphate, Polymyxin B, Oxolinic acid) classes as shown in Table-2.

Table-2: Antibiotic trail against *Clostridium perfringens* isolated from bovine fecal samples.

Class	Antibiotics	Abbreviation	ugs	Zones (mm)
Chloramphenicol	Chloramphenicol	C	30	25mm
Penicillin	Amoxicillin	AML	10	27mm
	Penicillin G	P	10	15mm
Tetracycline	Tetracycline	TE	30	27mm
Polypeptides	Colistine sulphate	CT	30	Resistances
	polymyxin B	POL	30	Resistances
	Oxolinic acid	OXA	10	Resistances
Glycopeptides	Vancomycin	VA	30	22mm
Quinolones	Ciprofloxacin	CIP	5	30mm
	Kanamycin	K	30	19mm
Aminoglycoside	Gentamycin	GN	10	21mm
	Streptomycin	STR	10	13mm
Flagyl	Metronidazole	MTZ	25	Resistances
Lincosamides	Lincomycin	MY	30	20mm
Macrolides	Erythromycin	E	15	22mm
Sulfonamides	Trimethoprim	W	5	Resistances

DISCUSSION:

Total 200 samples were collected out of which 74% showed positive to *Clostridium perfringens* while 26% were negative. The age wise distribution for enterotoxeamia was 20 % in 6 month, 16 % in 1year, 13% in 2 years, 11% in 3 years, 8% in 4 years and 6% in 5 years. The incidence of enterotoxaemia in spring and autum were more as compared to other seasons. The cattle of kacchi district were 54% and buffaloes were 20% effected to enterotoxaemia. The buffalo of kacchi district was less affected then cattle. Different antibiotic result showed that *Clostridium perfringens* were sensitive to Chloramphenicol, Penicillin, Tetracycline, Glycopeptides, Quinolones, and Aminoglycoside classes our finding was same as reported by Rahaman *et al* [12]. The sign and symptoms such as kicking bally due to colic pain, diarrhea, anorexia and muscle tremors were observed. While on postmortem the necrotic enteritis, hemorrhages in vital organs like pulpy kidney and liver were observed. Our finding were same as described by Nasir *et al* [13].

The present study demonstrated the PCR method was found highly effective diagnostic approach for rapid and trust worthy diagnosis of enterotoxeamia. The PCR showed clear bands of 233pb of CPE gene of *Clostridium perfringens*.

CONCLUSION:

During the samples collection from different areas of Kacchi district.it is observed that farmer`s having unawareness and lack of knowledge about necrotic enteritis in bovine. We should control the disease by regular vaccination and proper feed intake management practices should be followed.

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