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Isolation and identification of *Clostridium perfringens* from milk samples and dairy products of Quetta City, Pakistan

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Abstract

The concern study was conducted to isolate the causal agent of food poisoning in milk samples and dairy products from various zones of Quetta city. Total 1120 samples were collected, out of that 19.28 % were positive for *Clostridium perfringens* while 80.71% were found negative. The zone wise distribution of positive cases shows 3.57% from east zone, 2.8% from west zone, 5.71% from north zone, and 7.14% from south zone of Quetta city. The milk and dairy products of south zone were highly infected with *Clostridium perfringens* as compared to all three zones of Quetta city. The confirmation of *Clostridium perfringens* was done through gram staining, various biochemical tests and PCR. The PCR result displayed clear band of 541 base pairs CPE gene under UV light. Different drugs result showed that *C. perfringens* was sensitive to Amoxicillin (23mm), Penicillin G (16mm), Vancomycin (24mm), Gentamycin (20mm), Streptomycin (15mm), Chloramphenicol (26mm), Ciprofloxacin (24mm), Kanamycin (18mm), Amikacin (14mm), while high resistant was shown against Cefatoxine Sodium, Lincomycin, Erythromycin, Trimethoprim, Metronidazole, Colistin Sulphate, Polymixin B, Oxolinic acid and Neomycin Sulphate.

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Introduction

Clostridium perfringens is an obligate anaerobic, gram-positive, spore- forming, rod shape and non-motile bacteria (Bohar *et al*., 2011). *Clostridium perfringens* is present in natural environment (Miah *et al*., 2011), and is a common contaminant of food (Tseng and Labbe, 2000).

It produces enterotoxin which is recognized as diarrhea genic toxin responsible for food-borne illness (Scallen *et al*., 2011). In food poisoning sign such as, abdominal cramps are followed by diarrhea within 6 to 24 hours (Doly *et al*., 2015). Milk is highly perfect food which contains all those nutrients which a body need (Waser *et al*., 2007).

There is substantial epidemiological evidence that consumption of raw milk during childhood may protect against asthma, allergies and other immune – mediated diseases but on the other hand it could be dangerous to health if infected.

It is perfect media for the growth of spore forming bacteria and can easily be contaminated by *Clostridium perfringens* (Addisu and Melesse, 2015).

The dairy products and milk are the most consumed foods used worldwide (Anil and Sangeeta, 2015). Milk is subjected to contamination from different sources, and these contaminates find their way to milk and its products through faulty methods of production and handling.

On the other hand, soil, air, water, bedding, feeds, excreta of human and animal act as important sources of contamination. Various studies have been conducted on this problem which shows that the spores are highly resistant against heat during processing of milk and their products.

The *Clostridium perfringens* is resistant to many antibiotics. Antimicrobial resistance could be associated with the extensive therapeutic use of antimicrobials or with their administration as growth promoter. Therefore, this study was carried out for the evaluation of bacteriological patterns of *Clostridium perfringens* as one of food poisoning microorganism in milk samples and dairy products of Quetta city.

Materials and methods

Milk and dairy products samples were collected in sterile container from different zones.

Collection of samples

Total 1120 samples of milk and dairy products were collected in this study. The samples were brought from various zones of Quetta city in sterile container with double volume of phosphate buffer solution (PBS) and transferred to laboratory in cold box for further laboratory processes at Center for Advanced Studies in Vaccinology and Biotechnology, University of Balochistan, Quetta for microbiological and molecular analysis.

Antibiotic sensitivity test

Standardized antibiotic sensitivity experiment was done on Muller Hinton agar by means of disc diffusion Bauer technique and Mcfarland turbidity standard technique (0.5) following CLSI procedure. isolates were measured as sensitive and resistant to specific antibiotic on the basis of inhibitory zone (Miyashiro *et al*., 2009).

Isolation and identification of Clostridium perfringens

Each sample of milk and dairy products were inoculated deep in sterile Robertson's cooked media broth (RCMB).

The tubes containing RCM and samples were kept in water bath for remove of non-spore forming aerobic bacteria and vegetative cells for a period of 10-15 at 80 ^{o}C after heat shock the tubes were incubated anaerobically at 37 ^{o}C for 24 hours in anaerobic jar. Next day a drop of sample from tubes applied on sterile agar plate for colonies morphology.

A suspect colonies of *Clostridium perfringens* then confirm through gram staining, various biochemical

tests, sugar fermentation tests and PCR.

Molecular characterization

Genomic DNA was extracted through thermo scientific genomic DNA purification kit. PCR was performed for amplification of expected toxin gene CPE (epsilon toxin). Two sets of primers of the following arrangement F (ACTGCAACTACTACTCATACTGTG), R (CTGGTGCCTTAATAGAAAGACTCC) were used for amplification of a 541-bp of the targeted CPE gene. PCR was carried out in 25 µl reaction mixture containing10.5 grade water, master mix 10.5(2×AmpMasterTM Taq), 2µl of extracted DNA and 1 µl of primers of each. After an initial denaturation at 94 ^{o}C 5 minutes, 25 cycles PCR amplification cycles consisting of denaturation at 94 ^{o}C for 1 minute, annealing at 55 ^{o}C for 1 minutes and an extension at 72 ^{o}C for 1 minute were performed followed by a final extension at 72 ^{o}C for 10 minutes. PCR products were then visualized using ethidium bromide stains at agrose gel (.5µl) electrophoresis.

Results and discussion

Total positive and negative results of clostridium perfringens

Among 1120 milk and dairy products samples 19.28 % were *Clostridium perfringens* positive and 80.71% were found negative as shown in fig -1.

Zone wise distribution of milk and dairy products

It was observed that out of positive samples north zone was 5.71%, south 7.14%, east zone 3.57%, and west zone was 2.8% positive for *Clostridium perfringens.*

Table 1. Clostridium perfringens confirmation through different biochemical tests.

S. No	Biochemical tests	*Clostridium perfringens*	
1	Gram staining	Gram positive	
2	Shape	Rod, Rectangular, Endospore (Sub terminal, Central)	
3	Size under microscope	Measure 0.3-2.0x1.5-10.0µm in size	
4	IMVIC	Indole test	-
		Methyl red test	+
		Voges-proskauer test	-
		Citrate test	-
5	Biochemical tests	Catalase test	-
		Oxidase test	-
		Urease test	-
		Motility test	-
		H_2S gas production test	+
		Gelatin liquefaction test	+
		Lacithinase test	+
6	Sugar Fermentation tests	Fructose	+
		Maltose	+
		Mannitol	-
		Dulcitol	-
		Glucose	+
		Lactose	+
		Dextrose	+
		Sorbitol	+
7	Selective Media(RCM)	Opaque white color, Smooth regular convex colonies appeared	

The south zone was highly infected with *Clostridium perfringens* among the three zones of Quetta city as shown in fig-2.

Prevalence of clostridium perfringens in milk and dairy products

The milk and dairy products result shows that milk was 8.01%, Cheese was 3.73%, Butter 2.74% and Yogurt was 4.71% affect with *Clostridium perfringens* as shown fig -3. The milk of Quetta city was highly infected as compared to dairy other products.

Table 2. Sensitivity and resistance of antibiotics against C.perfringens.

Class	Antibiotics	Abbreviations	µgs	zone(mm)	
Chloramphenicol	Chloramphenicol	C	30	26	
Penicillin	Amoxicillin	AML	10	23	
	Penicillin G	P	10	16	
Polypeptides	Colistin Sulphate	CT	10	00	
	Polymixin B	POL	30	00	
	Oxolinic acid	OXA	30	00	
Glycopeptides	Vancomycin	VA	10	24	
Quinolones	Ciprofloxacin	CIP	5	24	
	Amikacin	AK	30	14	
	Kanamycin	K	30	18	
Aminoglycosides	Gentamycin	GN	10	20	
	Streptomycin	STR	10	15	
Flagyl	Metronidazole	MTZ	25	00	
Cephalosporin's	Cefatoxine sodium	CTX	30	00	
Liconsamides	Lincomycin	L	30	00	
Macrolides	Erythromycin	E	15	00	
Sulfonamides	Trimethoprim	w	5	00	

Biochemical characterization of clostridium perfringens

On the basis of isolation and identification of *Clostridium perfringens* various methods adopted such as using selective media, microscopic examination, specific colony characters, staining technique, PCR based technique, and various types of biochemical tests (sugar fermentation tests, catalase, oxidase, urease, gelatins liquefaction, lecithinase tests, and IMVIC) as shown in Table-1

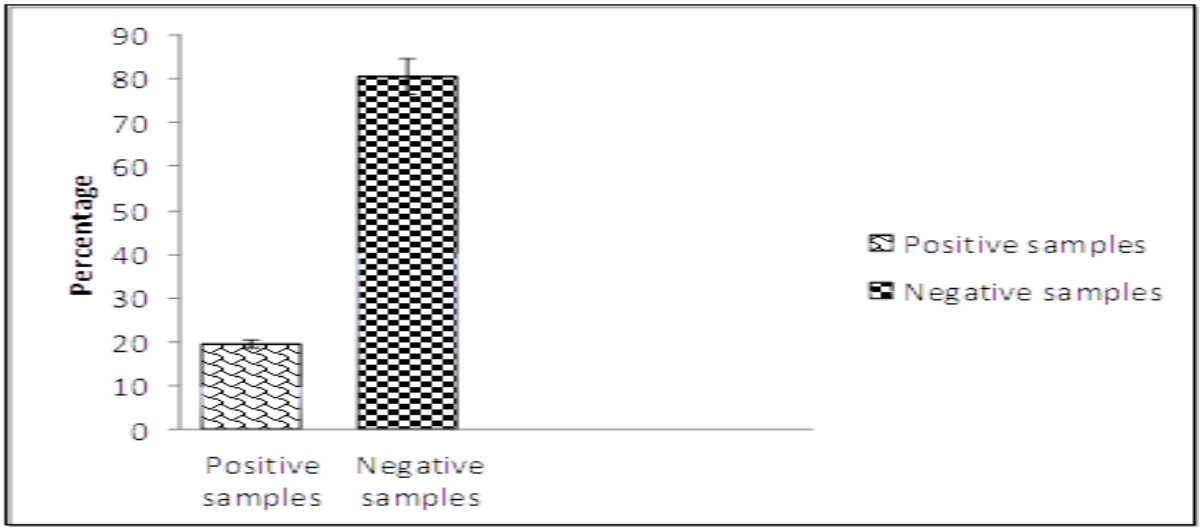

Fig. 1. Positive and negative samples of Clostridium perfringens isolated from Quetta city.

Antibiotic sensitivity test

The *Clostridium perfringens* was sensitive to Streptomycin (15mm), Penicillin G (16mm), Gentamycin (20mm), Amoxicillin (23mm), Chloramphenicol (26mm), Vancomycin (22mm), Ciprofloxacin (24mm), Kanamycin (18mm) and Amikacin (14mm). While *C. perfringens* was highly resistant to Cefatoxine Sodium, Colistin Sulphate, Polyxin B, Lincomycin, Trimethoprim, Erythromycin, Tetracycline, Neomycin Sulphate and Metronidazole.

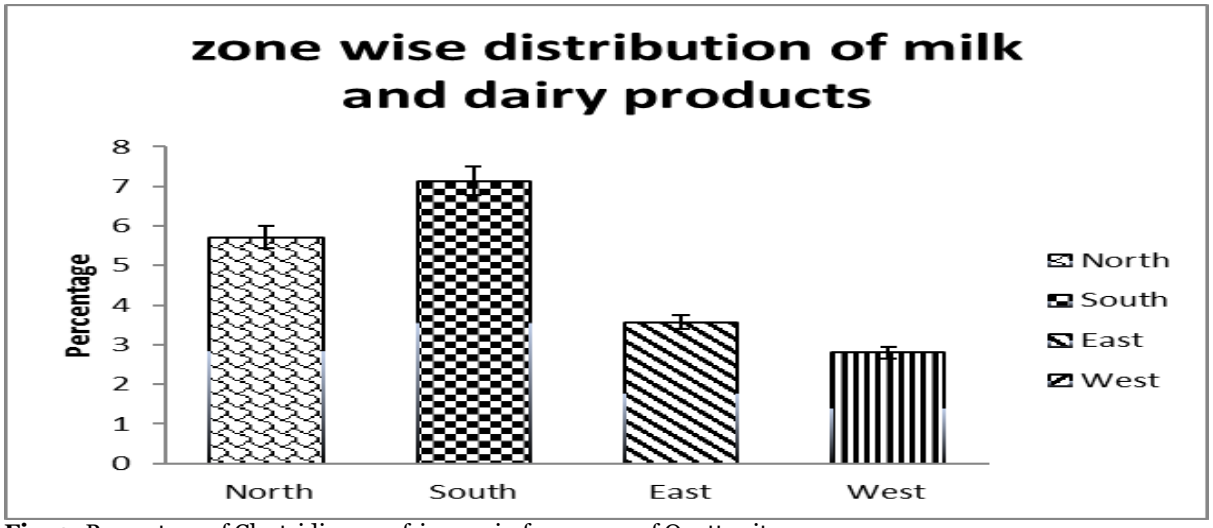

Fig. 2. Percentage of Clostridium perfringens in four zones of Quetta city.

Molecular study of clostridium perfrngens

The PCR based result showed clear bands of target (CPE) gene of 541-base pair after visualization using ethidium bromide for staining as shown in fig-4**.**

Discussion

Total 1120 samples of milk and dairy products were collected out of that 19.28% showed positive for *Clostridium perfringens* while 80.71% were found negative. The zones wise distribution of positive cases of milk and dairy products were 3.57% from east, 2.85%, from west, 5.71% from north and 7.14% from south zone of Quetta city.

The various zones result showed that south was highly infected as compare to other zones of Quetta city.

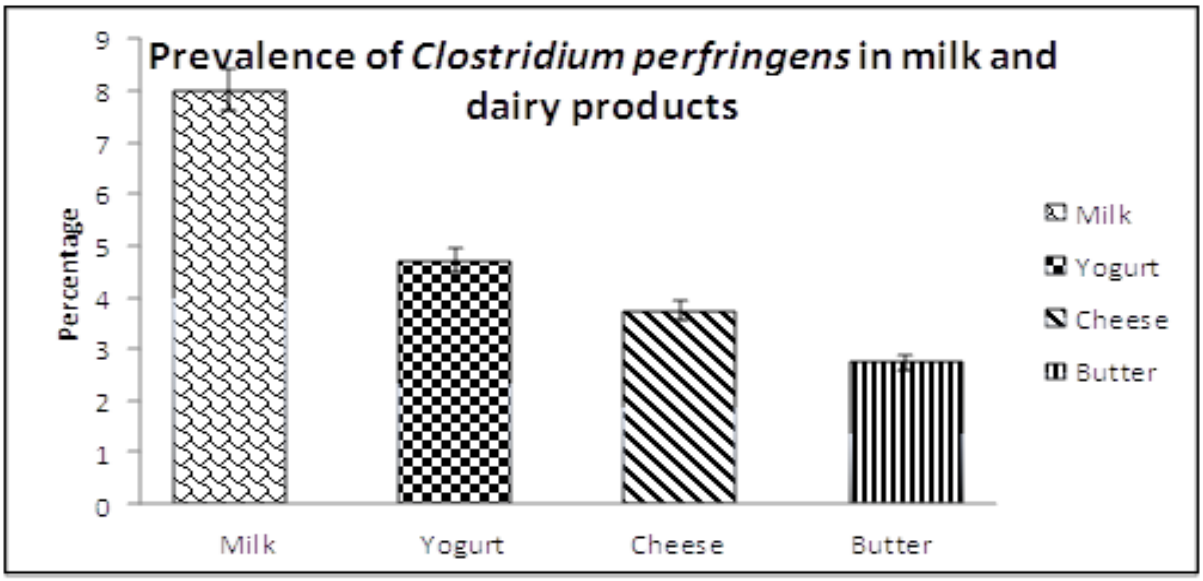

Fig. 3. Prevalence of Clostridium perfringens in milk and dairy products.

The milk and dairy products result showed that milk was 8.01%, Cheese was 3.73%, Butter 2.74% and Yogurt was 4.71% affected with *Clostridium perfringens*. The milk of Quetta city was highly infected as compared to other dairy products. All the biochemical and morphological tests were found same as formulated by phukan *et al.,* 1997.

The PCR based study was found highly authentic diagnostic approach for the accurate diagnosis of concern food poisoning disease. After visualization clear bands of targeted 541 bps CPE gene of *Clostridium perfringens* were appeared. The result of PCR was found same as formulated by Rowayda Osama *at el.*, 2015.

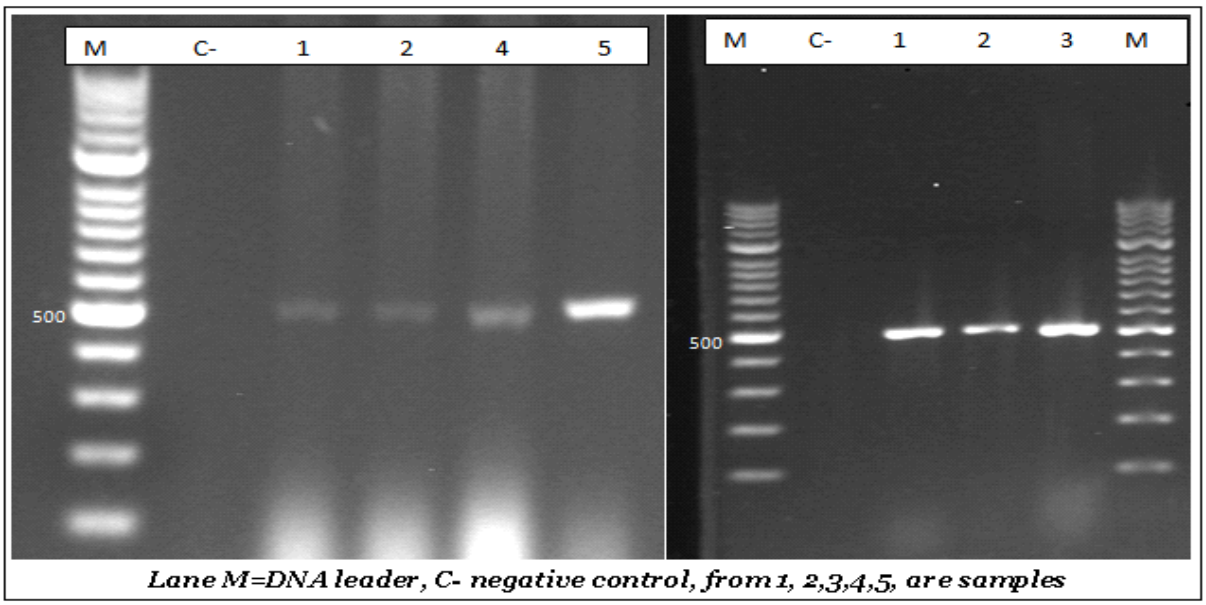

Fig. 4. Molecular identification of clostridium perfringens from milk and dairy products by using direct apply of gene primer specific.

Antibiotic sensitivity test was used which showed that *Clostridium perfringens* was highly sensitivity to different classes of drugs, Penicillin, Quinolones, Aminoglycosides, Glycopeptides and Chloramphenicol, while for others classes of drugs *Clostridium perfringens* was resistant such as, Polypeptides, Flagyl Cephalosporin's, Lincosamides, Macrolides, and Sulphanamides our results were found same as the tests performed by Osman and elhariri, 2013.

Conclusion

Present study manifested that during processing of milk and dairy products, necessary care is not undertaken and there is lack of awareness involving spores of *Clostridium perfringens* which can survive per long period of time, besides these, also there is lack of information concerning source of contamination of milk and dairy products. *Clostridium perfringens* can easily contaminate milk and its products due to the wide spread of spores which has been proved through this conducted research study.

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