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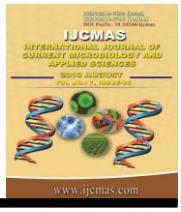

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Enterotoxigenecity and Typing of *Clostridium perfringens* Isolated from Raw Meat, Meat Products and Water Samples in Kashmir, India

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A B S T R A C T

The objective of the study was to see the prevalence of *C. perfringens* as well as to detect the enterotoxigenic genes in *C. perfringens* strains isolated from different meat and water samples in Kashmir, India. For this 275 different meat and meat product, and water samples were analyzed by standard bacteriological technique. All the Fifty nine (59) isolates recovered from these samples were typed according to their gene by multiplex PCR assay. Out of these, 44 (74.57%) isolates were confirmed as *C. perfringens* type A based on the presence of alpha toxin gene (*cpa*). The highest number of type A isolates were recovered from raw mutton (08) followed by mutton kabab (07) and mutton curry (06). Eight isolates (18.60%) were classified as un-typable strains as no other gene was detected by PCR. To ascertain the prevalence of *C. perfringens* enterotoxin gene (*cpe*), all type A isolates were also screened for presence of *cpe* gene separately. From the PCR assay, it was found that 13 (29.55%) of *C. perfringens* type A isolates carried the *cpe* gene of 233 bp. Isolates recovered from chicken pickle showed highest occurrence (100%) of *cpe* gene followed by raw chicken (50%) and mutton kabab (42.86%). Strain having the *cpe* gene is of public health concern that is mainly responsible for causing food poisoning.

Introduction

Clostridium perfringens is one of the most widespread pathogenic bacteria in the environment (Aberle *et al.*, 2001). The organism is responsible for several important enteric diseases, quite apart from its leading role as the cause of numerous different histotoxic infections both in humans and animals (Rood and Cole, 1991; Smedley and Mc Clane, 2004). Food-borne illness is caused by *Clostridium perfringens* throughout the world resulting from the consumption of contaminated food (Lin and Labbe, 2003). The organism is ubiquitously found in natural environment including soil, water source as well as in the intestinal tract of humans and animals and gains easy access to different foods and spices (McClane, 2001; Hörman *et al.*, 2004; Aguilera *et al.*, 2005). Several studies reported that meat and meat products, including poultry meat are the most common food vehicles of 'Clostridial' food poisoning (Hatakka and Halonen, 2000; McClane, 2001). The common occurrence of this organism in raw meat and retail food may be due to the

contamination of the carcasses and meat with the intestinal contents of the animals during the slaughtering and evisceration process (Lin and Labbe, 2003; Wen and McClane, 2004). However, much less is known about the reservoirs for *cpe*-positive *C. perfringens* type A strains (Heikinheimo and Korkeala, 2005).

In Kashmir several meat products like mutton kabab, mutton rista, yakhni, chicken kabab etc. known as 'Kashmiri Wazwan' are very popular because of their delicious preparations. However, information regarding the microbiological quality of these products especially in relation to *C. perfringens* type A is very scanty in this region. So, the investigation was carried out to see the prevalence of *C. perfringens* type A in different animal products and to find out the sources of contamination of these foods, so that preventive measures can be taken.

Materials and Methods

Isolation and identification of *Clostridium* species

A total of two hundred seventy five (n=275) samples comprising raw meat, meat products like mutton kabab, chicken kabab, mutton curry, chicken curry, mutton pickle and mutton rista (traditional Kashmiri dish), and water samples used for washing of carcasses were collected randomly from butcher shops, grocery stores and restaurants in different areas of Srinagar city in India. The samples were collected in sterile plastic zip lock sachet, brought to the laboratory on ice and processed immediately as per standard microbiological methods described by Food and Drug Administration (1998) and Holt *et al.,* (1994). Briefly, 25 g of samples were blended in sterile blender with 225 ml peptone water and then 1.0 ml solution is plated in sterile egg-yolk free tryptose-sulfite-cycloserine (TSC) agar (Hi-Media) and incubated at 37°C for 24 hrs under anaerobic conditions using McIntosh jar with Anaero-gas packs (Hi-media). Colonies from egg yolk free TSC agar plates were then inoculated into Fluid Thioglycollate (FTG) medium (Hi-Media) and incubated for 24 h at 37°C. A loopful of each thioglycollate culture medium was streaked on TSC agar containing egg yolk (Hi-media) and the plates were incubated in anaerobic jar for 24 h at 37°C. Colonies with gray opaque zones caused by lecithinase activity were considered positive for *C. perfringens*. The isolates were further confirmed by Gram's staining, nitrate reduction, gelatin liquefaction, motility in motility-nitrate medium and fermentation of glucose and lactose. All confirmed isolates of *C. perfringens* were maintained in Cooked meat medium (CMM) (Hi-Media) for further studies.

Characterization of *C. perfringens*

Isolates of *C. perfringens* were screened for presence or absence of alpha toxin gene (*cpa*), beta toxin gene (*cpb*), epsilon toxin gene (*etx*) and iota toxin gene (*iA*) by multiplex PCR using specific primers (Table 1) as per the method of Songer and Meer (1996) and typing was done accordingly.

Preparation of DNA template

The template DNA was prepared by boiling and snap chill method. Cultures maintained in CMM were inoculated in 6 ml FTG medium and incubated at 37°C for 18 hr. One (1.0) ml of broth culture was centrifuged by centrifugation at 5000 rpm for 3 minutes. Cells were collected in micro-centrifuge tubes, pellet was washed once with 500 µl sterile double distilled water and re-suspended in 100 µl sterile double distilled water. The micro-centrifuge tubes were kept in boiling water bath for 10 min and then in crushed ice for 20 min. The chilled samples were centrifuged at

6000 rpm for 5 min and the supernatant was collected which served as template. The DNA extracted from isolates was stored at -20^{o}C till further use.

Detection of toxigenic genes

The multiplex PCR was performed in a final reaction volume of 50 μl containing 10 μl of template DNA, 5 μl of 1X PCR buffer (10 mM Tris-HCl pH 8.3 and 50 mM KCl), 1.5 mM $MgCl_2$ 3μl, 0.4mM of each dNTPs 0.4 μl, 0.5 μl of 0.2 μM Taq DNA Polymerase (MBI-Fermentas, Mumbai, India) and 1 μl of 0.2 μM of each primer. Amplification reactions were carried out in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Singapore) with initial denaturation at 94°C for 4 min followed by annealing of 30 cycles at 55°C for 2 min, extension at 72°C for 3 min and a final extension of 72°C for 4 min. The amplicons were separated on agarose gel (2.0%) stained with ethidium bromide (0.5 μg/ml), applying 5–6 Volts cm _1, and sizes were estimated using 100 bp DNA ladder (MBI-Fermentas).

Detection of enterotoxin gene (*cpe*)

Detection of *C. perfringens* enterotoxigenic gene (*cpe*) was carried in all type A strain by PCR with specific primers as per the method of Songer and Meer (1996) as mentioned above and the specific forward and reverse primer pairs for *cpe* gene of 233bp were 5$^{/}$-GGAGATGGTTGGATATTAGG-3$^{/}$ and 5$^{/}$-GGACCAGCAGTTGTAGATA-3$^{/}$ (Czeczulin *et al.,* 1993).

Results and Discussion

In the study, a total fifty nine (59) *Clostridium perfringens* isolates were recovered, of which 43 isolates from meat products and 16 from water used for washing carcasses (Table 2 and 3). The isolates were identified as *C. perfringens* on the basis of common morphological, cultural and biochemical characteristics along with ATCC -13124 strains (Table 4).

All these isolates were typed on the basis of presence or absence of toxigenic genes (*cpa, cpb, etx* and *iA)* detected by multiplex PCR. Out of 43 isolates recovered from meat and meat products, 35 (81.40%) were confirmed as type A which possesses alpha toxin gene (*cpa*) of 324 bp size of DNA (Plate 6). None of the isolates contain any of *cpb*, *etx* and *iA* genes as the product failed to amplify for these genes. Highest (08) number of type A isolates were found in raw mutton followed by mutton kabab (07) and mutton curry (06). Eight isolates (18.60%) were classified as un-typed strain as the strain unable to amplify any of the genes in PCR. Out of 16 isolates recovered from water, 09 (56.25%) were typed as type A. In an investigation, Elham and Nahla (2011) found that among toxigenic types, type A was the most predominant type of *C. perfringens* (46.8%) compared to type D (19.5%) and mixed types (23.3%) in the Egyptian meat products. PCR has been widely used in identifying the representative toxin genes of *C. perfringens* because of its high sensitivity (Songer and Meer, 1996). Many surveys on the incidence of *C. perfringens* in raw and processed foods have been conducted without regard of whether the isolates were enterotoxigenic or not (Eman *et al.,* 2007). In the current study, to ascertain the prevalence of *cpe* positive strains, all type A isolates were again examined for *cpe* genes by PCR. From the PCR assay, it was found that 11 (31.43%) type A isolates from meat and meat products found having *cpe* gene of approximately 233 bp product size (Plate 7). Highest number of *cpe* positive strains (03 of 07) was found in mutton kabab (Table 2). Two isolates (22.22%) recovered from water were also confirmed as *cpe* positive strains by PCR (Table 3).

Table.1 Specific primers used for the detection of *C. perfringens* toxin genes

Target genes	Primer sequences (5′-3′)	Amplicon size (bp)	Reference
Cpa	For 5′-GCTAATGTTACTGCCGTTGA-3′ Rev 5′-CCTCTGATACATCGTGTAAG-3′	324	Titball *et al.*, (1989)
Cpb	For 5′-GCGAATATGCTGAATCATCTA-3′ Rev5′-GCAGGAACATTAGTATATCTTC-3′	180	Hunter *et al.*, (1993)
Etx	For 5′-GCGGTGATATCCATCTATTC-3′ Rev 5′-CCACTTACTTGTCCTACTAAC-3′	655	Hunter *et al.*, (1992)
Ia	For 5′-ACTACTCTCAGACAAGACAG-3′ Rev 5′-CTTTCCTTCTATTACTATACG-3′	446	Perelle *et al.*, (1993)

For: Forward, Rev: Reverse

Table.2 Typing of *C. perfringens* isolates by PCR recovered from meat and meat products

Type of Samples	No. of *C. perfringens* isolates	*C.perfringens* types			
		Type A (%)*	Untypable (%)*	*cpe* positive type A (%)**	*cpe* negative type A (%)**
Raw Mutton	8	8 (100.0)	0	2 (25.0)	6 (75.0)
Raw Chicken	6	4 (66.67)	2 (33.33)	2 (50.0)	2 (50.0)
Mutton kabab	9	7 (77.78)	2 (22.22)	3 (42.86)	4 (57.14)
Chicken kabab	5	3 (60.0)	2 (40.0)	1 (33.33)	2 (66.67)
Mutton curry	7	6 (85.71)	1 (14.29)	1 (16.67)	5 (83.33)
Chicken curry	2	2 (100.0)	0	0	2 (100.0)
Mutton pickle	4	4 (100.0)	0	1 (25.0)	3 (75.0)
Mutton rista	2	1 (50.0)	1 (50.0)	1 (100.0)	0
Total	**43**	**35 (81.40)**	**8 (18.60)**	**11 (31.43)**	**24 (68.57)**

*% Calculated according to the number of *C.perfringens*

** Calculated according to the number of *C.perfringens* type A

Table.3 Typing of *C. pefringens* isolates by PCR recovered from water

Type of Samples	No. of *C. perfringens* isolates	*C.perfringens* types			
		Type A (%)*	Untypable (%)*	*cpe* positive type *A* (%)**	*cpe* negative type *A* (%)**
Lamb washing water	6	5 (83.33)	1 (16.67)	1 (20.0)	4 (80.0)
poultry washing water	3	2 (66.67)	1 (33.33)	0	2 (100.0)
Scalding tank water	7	2 (28.57)	5 (71.43)	1 (50.00)	1 (50.0)
Total	**16**	**9 (56.25)**	**7 (43.75)**	**2 (22.22)**	**7 (77.78)**

*% Calculated according to the number of *C.perfringens*
** Calculated according to the number of *C.perfringens* type A

Table.4 Biochemical characteristics of *C. perfringens* isolates from meat, meat products and carcass washings

No.	Name of the test	Characteristics	ATCC-13124	Field isolates
1	Growth on selective TSC agar (without egg yolk)	Demonstration of black colonies (Plate 1)	+ +	+ +
2	TSC (with egg yolk)	Lecithino-vitaline reaction (Plate 2)	+	+
3	Gram staining	Gram positive large rods (Plate 3)	+	+
4	Motility test (motility nitrate medium)	Motility	-	-
5	Malachite green staining	Presence of spores	+	+
6	Litmus milk test	Stormy fermentation with gas (Plate 4))	+	+
7	Blood agar (5% sheep blood)	β-haemolytic, double zone of haemolysis (Plate 5)	+	+
8	Nitrate reduction test	Reduction of nitrates to nitrites	+	+
9	Gelatin liquefaction test	After 48 hours	+	+
10	Carbohydrate fermentation			
		Glucose	Acid and gas	+ +
		Lactose	Acid and gas	+ +
		Maltose	Acid and gas	+ +

Plate.1 Typical black colour colonies of *C. perfringens* on tryptose sulfite-cyclosterin (without egg yolk) agar

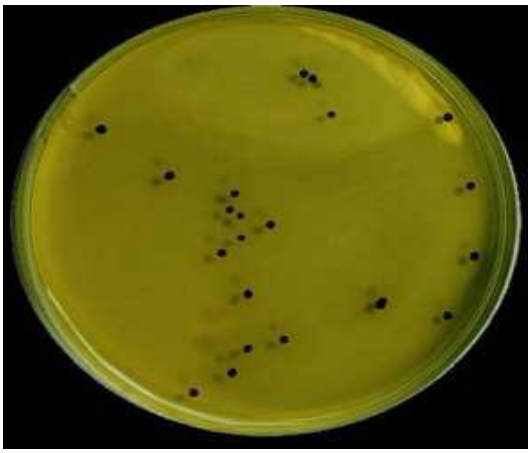


Plate.2 Colonies of *C. perfringens* on tryptose-sulfite-cycloserine (with egg yolk) agar showing lechithinase activity

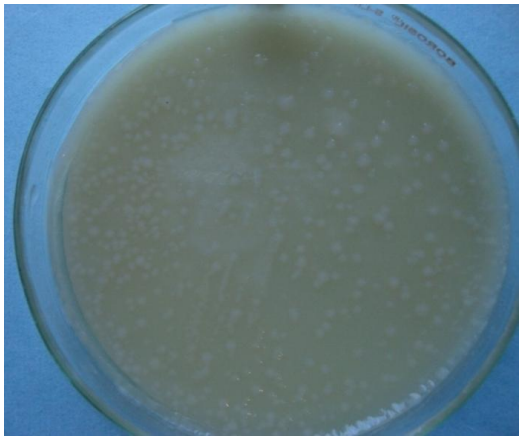


Plate.3 Gram positive large rods of *C. perfringens* (100x)

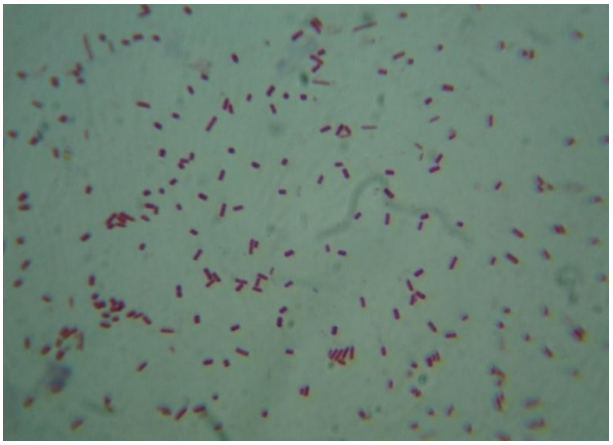
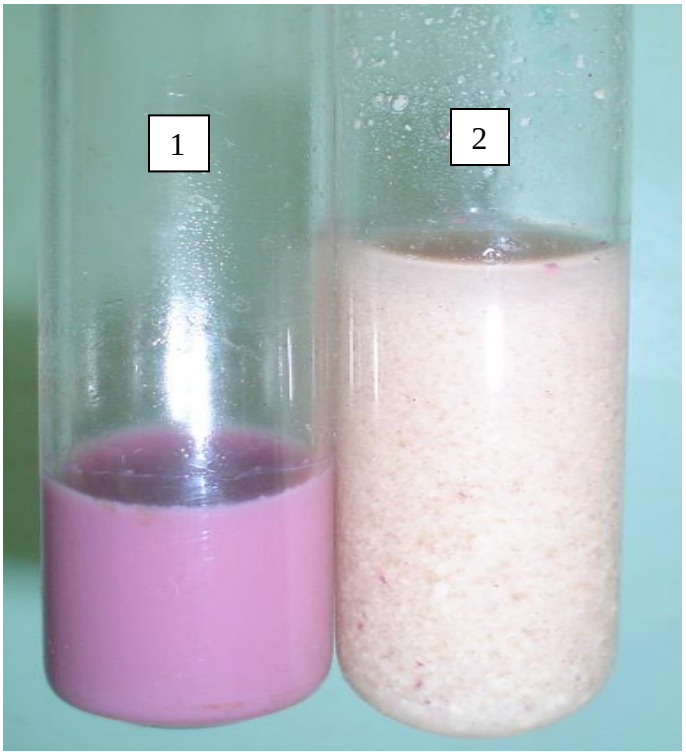


Plate.4 Stormy fermentation produced by *C. perfringens* in litmus milk



Tube-1: Negative reaction Tube-2: Positive reaction

Plate.5 *β* haemolysis produced by *C. perfringens* type A in 5% sheep blood agar

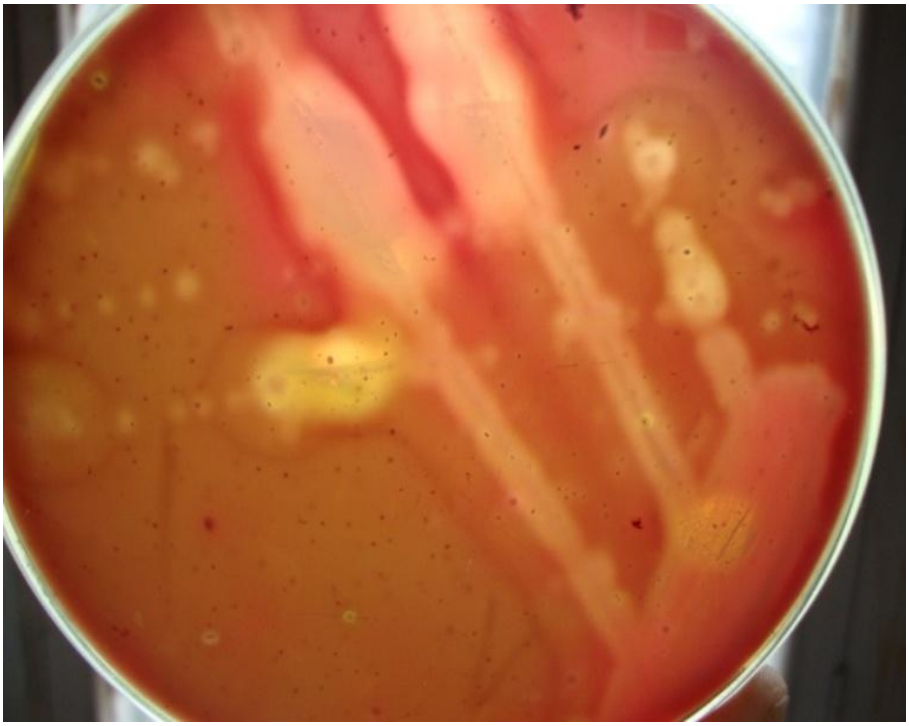
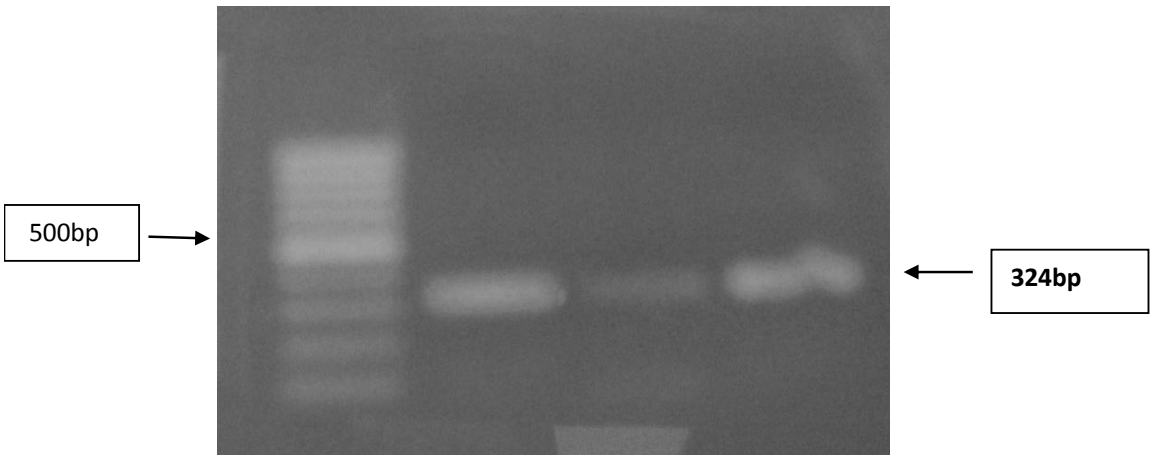
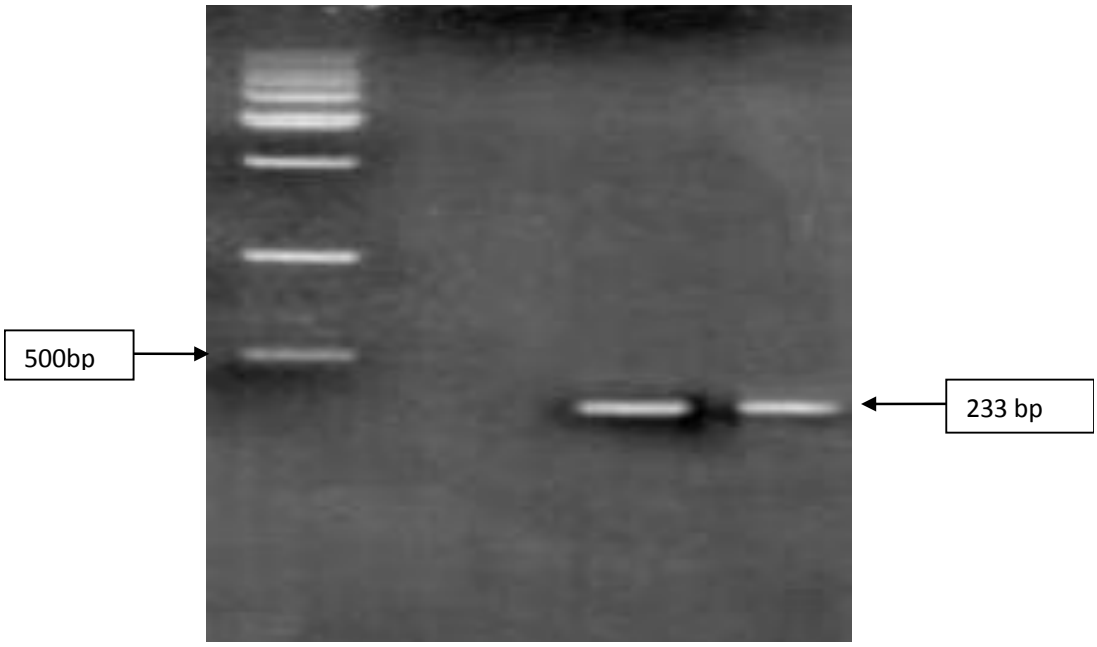


Plate.6 Bands revealing amplification of *C. perfringens* alpha toxin gene (*cpa*) by PCR



Lane 1: Standard molecular weight marker ladder (100 bp)
Lane 2, 3 and 4: Amplified *C. perfringens* alpha toxin gene (*cpa*) at 324 bp

Plate.7 Bands revealing amplification of *C. perfringens* entero-toxin gene (*cpe*) by PCR



Lane 1: Standard molecular weight marker ladder (100 bp)
Lane 2: Negative sample
Lane 3 and 4: Amplified *C. perfringens* entero-toxin gene (*cpe*) at 233 bp

Earlier many investigators reported the presence of enterotoxin gene in *Clostridium perfringens* type A isolates, where both alpha and enterotoxin gene were detected by PCR (Czeczulin *et al.*, 2005; Eman *et al.*, 2007; Singh *et al.*, 2005). Singh *et al.*, (2005) reported that 9.3, 32.4 and 15.5% isolates of *C. perfringens* from buffalo, goat and poultry meat respectively possessed the entero-toxin gene (*cpe*). However, Lin and Labbe (2003) could not detect any *cpe* genes in 133 food samples and concluded the rarity of *cpe* positive strains in retail foods and the genetic diversity among non-outbreak strains in

United States of America. Recovery of higher percentage (31.43%) of *cpe* positive *C. perfringens* type A isolates from meat and meat products than global average (5%) is of serious concern in respect of public health. Daube *et al.,* (1996) and Songer and Meer (1996) have indicated that less than 5% of the global *C. perfringens* population recovered from various sources only carries the entero-toxin gene (*cpe*).

In the study *C. perfringens* was found as a common contaminant of meat and meat products. Excessively contaminated meat and meat products with this microorganism are undesirable from public health point of view. Prevalence of toxigenic genes (*cpe*) in some type A isolates is epidemiologically significant which explain the association of the bacterium in food poisoning cases in this region. Recovery of higher percentage (31.43%) of *cpe* positive *C. perfringens* type A isolates in the study than global average (5%) is of a serious concern with respect to public health. As typing procedure like toxin neutralization test and animal inoculation test consumes a lot of anti-sera requiring experimental animal, PCR can be used to determine the presence of toxin genes and for typing of *C. perfringens* accordingly. This technique also gives the opportunity to type the isolates that could not be typed by toxin neutralization test.

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