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Characterization of *Clostridium perfringens* of animals, birds, and foods of animal origin

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Abstract
Screening of 119 samples of clinically affected animals/birds and foods of animal origin revealed *C. perfringens* in 43 samples. Toxin typing of the isolated *C. perfringens* revealed 39 as toxin types A, while the remaining four were recognized as the new toxin type (type F). All the isolates irrespective of toxin type were haemolysin producing, while phospholipase C activity and DNase production were detected in 81.4 and 76.74 percent of the isolates, respectively. An identical plasmid of 23.1 kb size was detected in 16 isolates, of which 15 were belonged to type A and the rest to type F. The present observation on the recovery of haemolysin, phospholipase C and DNase producing isolates of *C. perfringens* type A, and particularly the type F from infected animals/birds as well as foods of animal origin could draw significant public health importance with a future study to correlate plasmid profiling with *C. perfringens* toxin types.

Keywords: *Clostridium perfringens*, toxin type A & F, haemolysin, phospholipase C, DNase, plasmid

Introduction
Amongst the species of Clostridium, *C. perfringens* is thought to be the most widely distributed pathogen on earth [7]. Spores of the organism persist in the soil, sediments and areas subjected to human or animal faecal pollutions [35]. *Clostridium perfringens* is capable of producing many important toxins and enzymes associated with numerous gastrointestinal infections, generically called enterotoxemias in most mammalian species and birds. Despite the controversial role, *C. perfringens* toxin type A is still frequently responsible for enteritis, abomasitis and/or enterotoxemia in cattle [23], horses [2], goats [10], and pigs [34]. With the discovery of pore forming toxins (NetB & NetF) in some strains of necrotic enteritis associated *C. perfringens* type A in poultry [18], Mehdizadeh Gohari *et al.* [24] could establish the important role of type A in animal disease. The major involvement of the small intestine of neonatal animals is usually observed in the other toxin types (B and C) [4]. With the inclusion of sporulation associated enterotoxin (CPE) as a major class of toxin for typing, *C. perfringens* has recently drawn great attention from the scientific community as an important agent associated with food born infection in humans [12]. Among the new toxin types, type F was confirmed to be responsible for human food poisoning as well as antibiotic associated diarrhea [30]. Despite the rare association, the severity of type C induced human intestinal infections, like necrotizing jejunitis, enteritis necroticans or pig-bel is more than type A infection [37]. With exception of gene encoding for alpha and theta toxin, many of the other extracellular toxins encoded genes are located on a large plasmid. The enterotoxin gene can be either chromosomal or plasmid located [29]. A detailed profiling of plasmid is a useful alternative epidemiological tool for differentiation of strains and typing of *C. perfringens*, during outbreaks [32]. Despite the biodefense and pathogenic importance, a little attempt has so far been made in India to explore the toxin-encoding *C. perfringens* virulence plasmids.

In addition to various chromosomal and plasmid encoded exotoxins, some virulence activities, like DNase production, haemolysin production, phospholipase-C activity are also produced by *C. perfringens.* These additional virulence activities are directly or indirectly be associated with the diseased conditions. Detection of these virulence activities may play an important role in the identification of pathogenic strains of *C. perfringens.*

Considering the public health importance of *C. perfringens*, an attempt was initiated to characterize *C. perfringens* isolates recovered from animals/birds infected with enteric infections, as well as from foods of animal origin, in respect to their toxin typing, phenotypic

detection of certain virulence factors, and their plasmid profiling.

2. Materials and Methods

2.1. Isolation of *Clostridium perfringens*

A total of 93 multi species faecal samples/ intestinal scrapings were collected randomly from organized and unorganized farms in and around Khanapara, Guwahati, Assam. Clinical samples were collected from cattle (47), goats (14), pigs (10), sheep (8) and broiler birds (14), clinically affected with diarrhea. Additionally another 26 food samples, comprising of raw cow milk (19) and broiler meat (7) were collected from local vendors.

Collected samples were screened for bacteriological isolation of *C. perfringens* by inoculating in to Robertson's Cooked Meat Broth (RCMB) tubes. Following heat treatment at 80°C for 10-15 min, inoculated RCMB tubes were incubated initially at 37°C under an anaerobic environment (Don Whitley anaerobic work station, UK). Incubated RCMB tubes exhibiting typical growth characteristics of *C. perfringens* were further sub-cultivated on sheep blood agar (5% v/v) with D-cycloserine (400 µg/ml) and further incubated at 37°C for 24 hours under anaerobic condition. Isolated hemolytic colonies (suggestive of *C. perfringens*) exhibiting non-spore forming Gram-positive bacilli were further subjected to molecular confirmation targeting species specific *cpa* gene (324bp).

2.2. Toxin typing of *C. perfringens* isolates

All the *C. perfringens* isolates were subjected to toxin typing, targeting the major toxin associated genes by uniplex PCR. Established primers of *cpa* (324bp), *cpb* (180bp), *etx* (655bp), *iA* (446bp), *cpe* (233bp) and *netB* (316bp) were used (Table 1). DNA was extracted from the respective *C. perfringens* isolates by the snap chill method. Briefly, suspension of 3-5 colonies in 100 ml nuclease-free water was boiled for 10 min, followed by snap chilling for 5 min. The suspension was then centrifuged at 12,000 rpm for 5 min and the supernatant was used as template DNA. The *cpa* positive culture (ATCC-13124) maintained in the laboratory was used as a positive control for initial confirmation of the isolates as *C. perfringens*.

The PCR amplification was carried out in 25µl reaction mixture prepared with 12.5µl of 2× PCR master mix (Thermo Fisher Scientific, USA), consisting of 4mM magnesium chloride, 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 0.05 units/ µl *Taq* DNA polymerase, reaction buffer, 0.5µM of each forward and reverse primers for respective gene and 3.0 µl of template DNA [9]. An additional 0.7µl of $MgCl_2$ was added to the reaction mixture and the final volume was made up to 25.0 µl with nuclease free water (NFW). PCR reaction was performed in gradient thermocycler (Techne, U.K) with previously reported cycling conditions (Table 2). Amplified products were subjected to agarose (1.5%) gel electrophoresis and visualized under Gel Doc system (MiniLumi, DNR Bio-Imaging System, Israel).

Table 1: Primer sequences used for toxin typing of *Clostridium perfringens*

Toxin genes	Gene sequence	Size (bp)	Ref.
cpa	F 5GCTAATGTTACTGCCGTTGA-3	324	Titball *et al.* (1989)
	R 5CCTCTGATACATCGTGTAAG-3		
cpb	F 5GCGAATATGCTGAATCATCTA-3	180	Hunter *et al.* (1993)
	R 5GCAGGAACATTAGTATATCTTC-3		
etx	F 5GCGGTGATATCCATCTATTC-3	655	Hunter *et al.* (1992)
	R 5CCACTTACTTGTCCTACTAAC-3		
iA	F 5ACTACTCTCAGACAAGACAG-3	446	Perelle *et al.* (1993)
	R 5CTTTCCTTCTATTACTATACG-3		
cpe	F 5GGAGATGGTTGGATATTAGG-3	233	Czeczulin *et al.* (1993)
	R 5GGACCAGCAGGTTGTAGATA-3		
netB	F 5-CGCTTCACATAAAGGTTGGAAGGC-3	316	Bailey *et al.* (2013)
	R 5-TCCAGCACCAGCAGTTTTTCCT-3		

Table 2: Thermal cycling conditions for amplification of major toxin associated genes in *C. perfringens* isolate

Step	Name of the target gene (s)	Temp.	Duration (minute)	Purpose	No. of cycle
1	*cpa*, *cpb*, *etx* and *iA*	94 ºC	4 mins	Initial denaturation	1
2		94 ºC	1 min	Denaturation	X 35
		55 ºC	1 min	Annealing	
		72 ºC	1 min	Extension	
3		72 ºC	10 mins	Final extension	1
1	*net*B	95 ºC	15mins	Initial denaturation	1
2		94 ºC	30sec	Denaturation	X 40
		53 ºC	90sec	Annealing	
		72 ºC	90sec	Extension	
3		72 ºC	10mins	Final extension	1

2.3. Phenotypic detection of certain virulence factors in *C. perfringens* isolates

All the isolates of *C. perfringens* recovered from various sources were investigated for phenotypic detection of deoxyribonucleases (DNase) production, haemolysin production, and Phospholipase C activity.

2.3.1. Haemolysin production

Detection of *in vitro* production of haemolysin by *C. perfringens* isolates was done by inoculating the respective bacterial colonies into sheep blood agar (5% v/v). Inoculated plates were incubated at 37°C anaerobically for 24 hr. and observe for the clear zone of haemolysis around the bacterial colonies.

2.3.2. Deoxyribonuclease (DNase) production
Detection of Deoxyribonuclease (DNase) production in the isolated *C. perfringens* of the different sources was carried out on DNase test agar. Briefly, a single pure colony of respective *C. perfringens* isolate was subjected to DNase agar plates (Hi-Media, India) supplemented with 0.3% yeast extract by spot inoculation. Inoculated plates were incubated anaerobically at 37°C for a minimum of 48 hr or until a suitable growth was obtained. Following incubation, the DNase plates were flooded with 1 N HCl, and the appearance of clear white zones surrounding the bacterial growth was considered as positive for DNase production.

2.3.3. Phospholipase C production
Isolated *C. perfringens* colonies were also studied for their *in-vitro* ability to produce phospholipase C. The test was carried out by spot inoculation of respective isolates into Wills and Hobb's medium (Hi-Media, India) with the incorporation of Egg Yolk Emulsion, skimmed milk, and rehydrated Willis and Hobbs supplement, as per manufacturer recommendation. Neomycin sulphate (250 µg per ml) was additionally added into the medium to inhibit the growth of *Bacillus* and *Staphylococcus*, and for reduction of coliform growth. The plates were incubated anaerobically at 37 °C for 24 hr. and observed for yellowish opalescence (Nagler's reaction) around the bacterial growth.

2.4. Plasmid profiling of *C. perfringens* isolates
All the isolated *C. perfringens* strains of different toxin types, recovered from different sources were evaluated with respect to the distribution of plasmid. Extraction of plasmid from the individual isolate was done by Gene Jet Plasmid Miniprep Kit (Thermo Fisher Scientific), as per manufacturer's instructions. The eluted purified plasmid was subjected to 0.8% agarose gel electrophoresis at 60 V for 60 min. The stained gel was visualized in the Gel Doc System (MiniLumi, DNR Bio Imaging System, Israel) and the image was captured.

3. Results
3.1. Isolation of *C. perfringens* from different sources and their toxin type(s)
Screening of 119 different samples, irrespective of source revealed 43 (36.13%) samples to be bacteriologically positive yielding an equal number of *C. perfringens* (Table 3). All the morphologically positive isolates were found to possess *cpa* gene of 324bp size (Fig.1). Among the *C. perfringesn* positive samples, 31 were belonged to faecal/intestinal scrapings of animals/birds with enteric infections, while 12 of the 26 food samples of animal origin exhibited *C. perfringens.*
Molecular investigation of all the isolates for major toxin associated gene(s) could reveal a majority of *C. perfringens* isolates as type A (39), bearing *cpa* gene (324bp) alone. The remaining four *cpa* positive isolates were identified as type F, possessing *cpe* gene with 233 bp size (Fig.2). Among the animal isolates, type A was recorded in infected cattle and sheep (7 each) and goats (3). None of the pig samples could reveal isolation of *C. perfringens.* Similarly, out of 14 isolates recovered from infected broiler birds, 11 were identified as toxin type A, while three isolates as type F. Toxin typing of *C. perfringens* isolates of food of animal origin revealed a high prevalence of type A, recovered from broiler meat (6) and raw cow milk (5). On the other hand, only one broiler meat sample exhibited toxin type F. The present study could not record *cpb* (beta toxin), *etx* (epsilon toxin), *iA* (iota toxin), *netB* (NetB toxin), and *cpb2* (beta2 toxin) in any of the *C. perfringens* isolates.

3.2. Phenotypic detection of certain virulence factors in *C. perfringens* isolates
An *in-vitro* study of all the 43 *C. perfringens* isolates for their ability to express certain virulence factors could reveal the production of haemolysin in all the 43 isolates, irrespective of toxin type and source of sample (Table 4 & Fig.3A). DNase production could be detected in 76.74% of the *C. perfringens* isolates, of which three belonged to toxin type F of clinically affected broiler (Table 4 & Fig.3B). However, the only type F isolate of chicken meat could not reveal DNase activity. Among the haemolytic *C. perfringens* isolates, 81.4% isolates including all the toxin type F exhibited Phospholipase C activity (Table 4 & Fig.3C).

3.3. Plasmid profiling of the *C. perfringens* isolates
Plasmid profiling of the 43 isolates of *C. perfringens*, both type A and F of various sources could exhibit plasmid of 23.1 kb in 16 (37.21%) isolates (Fig.4). Among the plasmid bearing isolates, 15 were belonged to type A, recovered from clinically affected animal/bird (sheep=7, cattle=3, goat=1, broiler bird=1), as well as broiler meat (3). The remaining one identical plasmid was detected in type F isolate, recovered from broiler meat (Table 4). No correlation could be made in respect to the distribution of plasmid in isolated *C. perfringens* toxin types with the phenotypic expression of virulence factor other than major toxins.

Table 3: Toxin types of *C. perfringens* recovered from infected animals/birds and food of animal origin

Nature of samples	Source	No. of samples screened for *C. perfringens*	No. of samples positive for *C. perfringens*	Toxin type	
				A (*cpa*)	F (*cpa* + *cpe*)
Faecal swab/ intestinal scrapings	Cattle	47	7	7	--
	Goat	14	3	3	--
	Pig	10	--	--	--
	Sheep	8	7	7	--
	Broiler Birds	14	14	11	3
Total		93	31	28	3
Food of animal origin	Broiler Meat	7	7	6	1
	Raw Cow Milk	19	5	5	--
Total		26	12	11	1
Total		119	43 (36.13)	39	4

Figure in parenthesis indicates the percentage

Table 4: Characterization of *C. perfringens* isolates in respect to Haemolysin, Phospholipase C, and DNase production and their plasmid profiling

Source	No. of isolates	Toxin type		No. of isolates +ve for			Detection of Plasmid (23Kb)
		A	F	Haemolysin	Phospholipase C	DNase	
Cattle	7	7	--	7	7	6	3
Goat	3	3	--	3	3	2	1
Sheep	7	7	--	7	5	6	7
Broiler Birds	14	11	--	11	9	9	1
		--	3	3	3	3	--
Broiler Meat	7	6	--	6	6	6	3
		--	1	1	1	--	1
Raw Cow Milk	5	5	--	5	1	1	--
Total	43	39	4	43 (100)	35 (81.4)	33 (76.74)	16 (37.21)

Figure in parenthesis indicates the percentage.

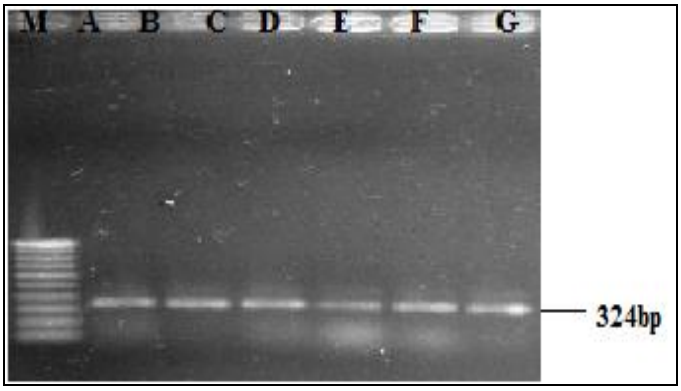

Fig 1: Amplification of *C. perfringens cpa* gene (324bp)

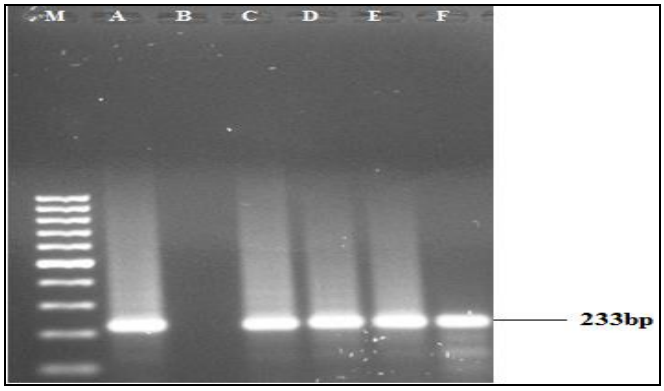

Fig 2: Amplification of *C. Perfringens cpe* gene (233bp)

Lane M	=	100 bp DNA ladder
Lane A	=	Positive control (ATCC-13124)
Lane B-G	=	Field isolates bearing *cpa* gene

Lane M	=	100 bp DNA ladder
Lane A	=	Positive control
Lane B	=	Negative control
Lane C- D	=	Field isolates depicting *cpe* gene (233bp)

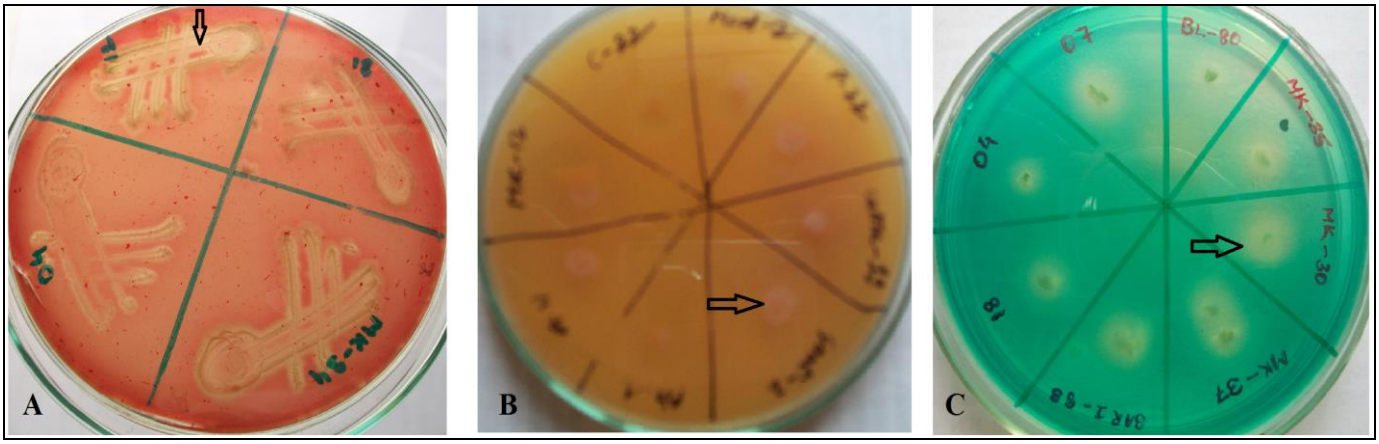

Fig 3: Phenotypic detection of certain virulence factors in *C. perfringens* isolates

A	=	Haemolysin production (Arrow indicates clear a zone of haemolysis on BA)
B	=	DNase production (Arrow indicates a clear white zone of DNase production)
C	=	Phospholipase C activity (Arrow indicates yellowish opalescence zone)

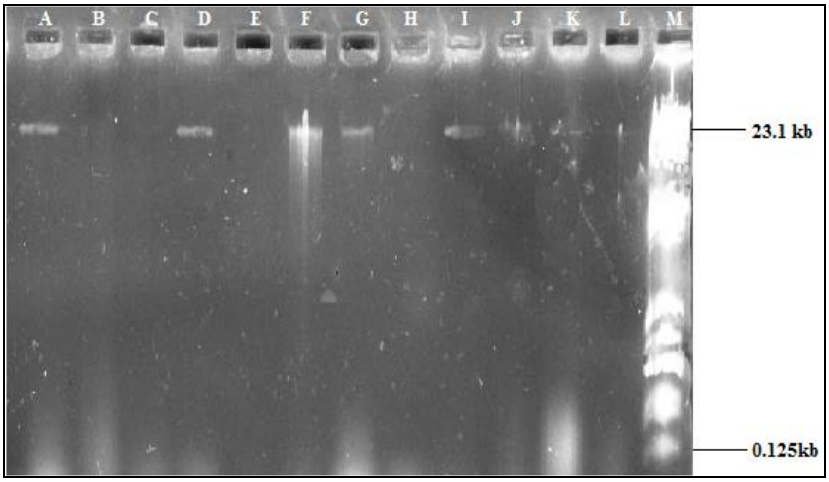

Fig 4: Plasmid profiling of *C. perfringens* isolates Lane A-L: Plasmid DNA from field isolates Lane M: λ-Hind III digest DNA ladder

4. Discussion

Clostridium perfringens, being associated with >20 virulent toxins have been responsible for intestinal diseases in both animals and humans throughout the past century [19]. The present observation on the isolation of *C. perfringens* from samples collected from a different source with major dominance of toxin type A was in agreement with many previous reports. Rahman *et al.* [28] recorded 47.06% of clinical samples irrespective of health status positive for *C. perfringens.* Contrary to the present observation on the isolation of *C. perfringens* from 33.33% of samples, Anju *et al.* [1] recorded *C. perfringens* only in 10.76% multi-species faecal and intestinal contents. Irrespective of health status, all the five toxin types of *C. perfringens* with major prevalence of type A are associated with animals and birds [13]. Recovery of a *C. perfringens* toxin type F from clinically affected broiler birds and broiler meat during the present study opined for the probable contamination of meat by *C. perfringens* type F, leading to human food poisoning. Human food poisoning is conventionally known to be associated with enterotoxin producing *C. perfringens* type F [29]. Enterotoxin producing Type A strains of *C. perfringens* associated with food poisoning has been known to be due to consumption of chicken meat [34,16]. Being ubiquitous and prevalence as poultry intestinal flora, chicken meat is usually contaminated with *C. perfringens*, resulting in human food poisoning [14, 5]. A study on the feasibility of *C. perfringens* as an index of fecal contamination of milk indicated a definite correlation between the hygienic condition of teat and udder, and the content of *C. perfringens* spores in the milk [24]. Although the association of *C. perfringens* type A with acute gangrenous mastitis in cow is rarely reported, the infected milk may be a potent source of human food poisoning [26]. In a similar study, Haque *et al.*[15] recorded few raw milk samples and Indian paneer samples positive for *C. perfringens* type A, of which one of the milk isolates was also found to possess enterotoxin (CPE) encoded *cpe* gene.

In addition to the toxins, *C. perfringens* produces certain virulence factors like DNase, haemolysis and phospholipase-C that may directly or indirectly contribute to the clinical presentation. The phospholipase C activity exhibited by *C. perfringens* is considered to be a major factor associated with the histolytic infection. Detection of in-vitro production phospholipase C by majority (81.4%) of *C. perfringens* isolates during the present study was a clear indication of in vitro expression of *cpa* gene by the majority of the isolates. Similar to present observation, a few lecithinase-negative variants of PLC-encoding *plc* positive *C. perfringens* was also previously recorded, which might be due to involvement of certain trans-acting factor in regulation of the *plc* gene expression [17]. The present study could not correlate expression of *cpa* gene for *in-vitro* release of phospholipase C with *C. perfringens* toxin type and their source.

Bacterial DNase facilitates the spread of infection by degrading the mesh of extracellular DNA of the host. The majority of the DNase-producing *C.perfringens* recovered from clinically affected animals and birds, during the present study was in agreement with the earlier findings [6, 34]. DNase activity exhibited by the majority of the *C.perfringens* isolates of clinically affected animals/birds might be an indication of the probable role of DNase in pathogenicity. No previous reports could be traced out from available literature in support of *in-vitro* DNase production by type A and F of *C. perfringens* of meat and milk sample.

The present observation on haemolysin production by all the *C. perfringens* type A isolates recovered from infected animals and birds might be an indication for the prevalence of alpha, delta and theta toxin, either alone or in combination, and their probable role in pathogenesis. Prevalence of haemolytic *C. perfringens* in diseased animals, recorded during the study was in agreement with previous reports on haemolytic *C. perfringens* strains of animal origin, both diseased and healthy [3, 27, 25].

Plasmid profiling has traditionally been used for strain differentiation of clinical isolates in the epidemiological investigation of *C. perfringens* [22]. Similar to the present observation on distribution of identical plasmid of 23.1 kb size in isolates of both type A and F, detection of plasmid within the range of 1.8kb - 45 kb size was also previously recorded in *C. perfringens* type C and D of goat with a suspected form of enterotoxemia. During that study, detection of *etx* and *cpb* genes could be correlated with the plasmid of type D (45 kb) and type C (37 kb), respectively [20]. The diffused patterns of plasmid DNA bands (23.1kb) seen in their study in both type C and D could opine that plasmids between 30 Kb to 45 Kb size might be mixed with the remnants of the chromosomal DNA. The present study could not correlate plasmid of 23.1kb size detected in *C. perfringens* with their respective toxin types. The *cpe* gene is usually found on the chromosome in strains that cause acute food poisoning, while *cpe* gene of plasmid origin is associated with non-food-borne diarrhea usually carries the *cpe* gene on a plasmid [8]. An

accurate plasmid profiling in relationship with *C. perfrringens* toxin type needs to have a suitable extraction method [32]. Earlier reports suggested that cells harvested from the late log phase and stationary phase gave poor results in plasmid extraction due to the problem of lyses of cells in *Clostridial* species [21, 36]. Plasmid isolation from saccharolytic Clostridial group was proved to be difficult due to DNA degradation by high activity of DNase [22]. Considering those previous reports on optimum plasmid isolation, a detailed study has to be carried out to develop an extraction procedure with consideration of bacterial growth phase to draw a conclusive remark on relationship plasmid profile with *C. perfringens* toxin type(s).

5. Conclusion

Clostridium perfringens, being a part of the normal intestinal flora of animals and birds, has drawn much attention from the scientific community because of its role as the major contributor to human food poisoning. Recovery of *C. perfringens* from 36.16% samples of infected animals/birds and foods of animal origin, during the present study, were an indication for distribution of *C. perfringens* in animal/bird environment and their association with enteric diseases. The present observation on the isolation of *C. perfringens* from meat and milk sold for human consumption could also be a great concern from a public health point of view. Despite the major distribution of toxin type A, the isolation of new toxin type F from infected broiler and broiler meat might be a significant finding for future study. *In-vitro* expression of haemolysin, Phospholipase C, and DNase in the majority of the isolates could correlate the association of *C. perfringens* isolates with intestinal infections in animals/birds and as a source for human food poisoning through food products of animal origin. However, no conclusive remark could be drawn in respect to the detection of an identical plasmid (32.1kb) in few isolates, both type A and F, and their relationship with *C. perfringens* toxin type (s). A further study has to be carried out to formulate a suitable plasmid extraction procedure in consideration of other factors.

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