



Isolation and Molecular Characterization of *C. perfringens* from Meat Samples

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ABSTRACT

The objective of the study was to observe the presence of *C. perfringens* in various meat samples and characterization of the isolates with molecular typing. The samples were collected from different retail outlets in Jabalpur city and processed aseptically. The samples were enriched in alternative thioglycollate medium followed by selective isolation in tryptose sulfite cycloserine agar. Out of 200 samples screened (50 each of raw buffen, chevon, chicken and fish meat), *C. perfringens* were isolated from 46 samples (23%). The highest presence of *C. perfringens* was found in buffen (28%), followed by fish (24%) and chevon and chicken (20% each). Lecithinase activity was shown by all the isolates of buffen, chevon and chicken, while, 11 (91.66%) isolates from fish were positive for lecithinase activity. Likewise, 36 (78.28%) isolates of *C. perfringens* displayed stormy fermentation in IMM within 14 – 18 hrs. The molecular study showed that all the isolates 46(100%) were found to harbor the 16S rRNA and lecithinase (*cpa*) gene, while none of the isolates displayed enterotoxin (*cpe*) gene. The presence of *C. perfringens* in raw meat indicated that the meat wasn't processed hygienically and poor sanitary conditions in retail outlets. Therefore, consumption of such meat may produce infectious disease and is a public health threat as many food poisoning strains of *C. perfringens* are heat resistant.

Keywords: Isolation, characterization, *C. perfringens*, meat

Meat and meat products are important source of high quality proteins with high biological value and essential amino acids that are required for body growth. Meat is the richest source of vitamin B, particularly thiamine, riboflavin and niacin. It also contains a good amount of fat soluble vitamin A, phosphorous, iron and copper (Sherikar *et al.*, 2013). India stands 5th in global production (6.3 million tons) and accounts for 3% of the total world meat production (220 million tons) (GOI, 2016).

Meat is often contaminated due to unhygienic production resulting in spoilage and food borne diseases. Food borne diseases occurs through consumption of raw meat or improperly cooked or contaminated with pathogens from sources such as water, raw ingredients, environment, food handlers and during processing of meat (Biddle *et al.*, 1993). Worldwide, food borne diseases are a major public

health burden leading to high morbidity and mortality. The global burden of infectious diarrhea involves 3 – 5 billion cases and nearly 1.8 million deaths annually, mainly young children, caused by contaminated food and water (NCDC, 2017). These deaths are due to contamination with virus, bacteria, parasites, toxins etc. The common bacteria found in meat and leads to infections are *Salmonella*, *Campylobacter*, *S. aureus*, *C. perfringens*, pathogenic *E. coli* etc (WHO, 2012).

C. perfringens is a Gram positive, anaerobe, spore-forming, mesophilic rod and is widely distributed in nature. It is generally associated with different forms of enterotoxaemia in animals and gas gangrene in animals and man (Singh and Bist, 2013; Das *et al.*, 2014; Yadav *et al.*, 2016). Based on their ability to produce certain exotoxins, *C. perfringens* are categorized in five types:



type A, B, C, D and E. The food poisoning strains belong to type A, and are generally heat resistant. These strains of *C. perfringens* exist in soil, water, foods, dust, spices and the intestinal tract of humans and other animals. The bacteria enter into meat directly from intestinal tracts of slaughtered animals or by the subsequent contamination of slaughtered meat from containers, handlers, or dust. It can withstand the environmental conditions of drying, heating and toxic compounds because of its spore forming ability. It is also a leading cause of food poisoning in many developed countries (Jay *et al.*, 2005; Gurmu *et al.*, 2013; Yadav *et al.*, 2016).

The foods involved in *C. perfringens* outbreaks are often due to consumption of preparations of meat or its by-products kept for longer period of time without refrigeration. When such preparations are rewarmed after cooking, the heat of such foods is presumably inadequate to destroy the heat resistant endospores, thereby providing endospores favorable conditions to germinate and grow (Jay *et al.*, 2005). The presence of *C. perfringens* in raw meat is a direct source of contamination and thus may precipitate food borne illness. Thus, the present study was aimed to investigate the presence of *C. perfringens* in different meat samples and their molecular characterization.

MATERIALS AND METHODS

Isolation

A total of 200 meat samples consisting 50 each of raw carabeef, chevon, chicken and fish were collected from different retail outlets in Jabalpur city. The meat samples were processed as early as possible in the laboratory. Isolation was performed with enrichment of sample in alternative thioglycollate broth followed by selective plating in tryptose sulphite cycloserine (TSC) as per the method described by Agarwal *et al.* (2003) and Singh *et al.* (2006). The meat samples were diluted (1:10) by taking 10 g sample in 90 ml normal saline followed by addition of diluted sample into alternative thioglycollate broth. The enriched sample was incubated at 37°C for overnight.

During selective plating, an agar overlay technique in tube was used using tryptose sulphite cycloserine (TSC) agar. Alternative thioglycollate enriched inoculum (0.1 ml) was taken in tube followed by addition of 10 ml of TSC agar. After 5 min, tube was overlaid with 2-3ml of TSC

agar and incubated at 37°C for 10-12h after solidification. Presumptive *C. perfringens* colonies (black colonies) from TSC was taken in alternative thioglycollate broth and incubated at 37°C for 10-15 hrs. The presumptive isolates of *C. perfringens* were microscopically and biochemically characterized on the basis of colony morphology, Gram's staining, motility test, lactose - gelatin liquefaction and nitrate reduction test according to method described by Agarwal *et al.* (2003).

The isolates were tested for lecithinase and lactose fermenting activity using TSC with egg yolk emulsion and iron milk medium, respectively. The lecithinase activity was observed by analyzing zone of opalescence around the colony. This was done by agar overlay method in petriplates under anaerobic condition. The anaerobic condition was created by candle jar method, in which a lighted candle was kept under jar and thus anaerobiasis was created (Agarwal *et al.*, 2003; Ananthnarayan and Paniker, 2013).

Stormy fermentation was observed by adding inoculums of overnight grown alternative thioglycollate culture in iron milk medium followed by incubation at 46°C in a water bath for 14 – 18 hrs. Stormy clot fermentation was observed as described by John *et al.* (1982).

Molecular Characterization

DNA was isolated from overnight grown culture in alternative thioglycollate using Quik-start protocol (Qiagen, Germany). In brief, 1.0 ml overnight culture of *C. perfringens* isolate was taken into eppendorf tube and centrifuged at 10000 rpm for 5 min. The cells were resuspend in 200 µl phosphate buffer saline (PBS) followed by addition of 20 µl proteinase K and 200 µl buffer AL. Mixing was done by vortexing and then 200 µl ethanol (96 – 100%) was added. The mixture was transferred into a DNeasy mini spin column followed by centrifugation twice at 8000 rpm for 1 min. The flow - through was discarded. DNA was eluted by adding 200 µl buffer AE to the center of spin column membrane and incubated for 1 min at room temperature (15-25°C) followed by centrifugation for 1 min at 8000 rpm.

The PCR reaction mixture (25 µl) contains DNA - 1µl, 10X Taq DNA polymerase buffer - 2.5 µl (20 mM Tris - HCl, pH 8.0, 1mM DTT, 0.1 mM EDTA, 100 mM KCl,

0.5% Nonidet P40, 0.5% Tween 20 and 50% glycerol and 20 mM MgCl₂), dNTP - 0.2 mM, each forward and reverse primer - 10 pmol, Taq DNA polymerase - 1 unit and rest milli Q water.

The amplification cycles were carried out in thermocycler (Bio-Rad) as per the method described by Yadav *et al.* (2016) with primer sequences as depicted table 1. The amplification cycles started with initial denaturation at 95°C for 5 min followed by 35 cycles each having denaturation at 95°C for 1 min, annealing at 52°C for 16S rRNA and 54°C for *cpa* (lecithinase gene) for 1 min and extension at 72°C for 1 min with final extension for 5 min at 72°C. The *cpe* (enterotoxin) gene reactions were carried out at annealing temperature between 50 - 60°C. At every screening a nucleic acid blank negative control were taken along with isolates to monitor the quality control of PCR conditions. On completion of PCR, amplified products were resolved in 1.5% agarose gel under UV transilluminator.

Table 1: Details of *C. perfringens* primers used for molecular characterization with PCR

Gene	Primer	Size	References
16S rRNA (F)	AAAGATGGCATCATCAT-TAAC	279 bp	Wu <i>et al.</i> (2009)
16S rRNA (R)	TACCGTCAT-TATCTTCCCCAA A		
<i>cpa</i> (F)	GCTAATGTTACTGCC-GTTGA	324 bp	Titball <i>et al.</i> (1989)
<i>cpa</i> (R)	CCTCTGATACATCGTG-TAAG		
<i>cpe</i> (F)	GGAGATGGTTG-GATATTAGG	233 bp	Yadav <i>et al.</i> (2016)
<i>cpe</i> (R)	GGACCAGCAGTTGTA-GATA		

RESULTS AND DISCUSSION

The results of the isolation study indicated that out of 200 samples examined, 23% (46 isolates) had *C. perfringens*, with buffen (28%), fish (24%) and chevon & chicken (20% each) as depicted in table 2. The biochemical profiles of the isolates are shown in table 2. The molecular study showed that all the isolates were found to harbor the 16S rRNA (Fig. 1) and lecithinase (*cpa*) gene (Fig. 2), while none of the isolates displayed enterotoxin (*cpe*) gene.

Table 2: Occurrence of *C. perfringens* in meat

Type of sample	Number of samples	Occurrence of <i>C. perfringens</i>	Lecithinase activity	Stormy fermentation in IMM
Buffen	50	14 (28.0%)	14 (100.0%)	10(71.4%)
Chevon	50	10(20.0%)	10 (100.0%)	7(70.0%)
Chicken	50	10(20.0%)	10 (100.0%)	8(80.0%)
Fish meat	50	12(24.0%)	11 (91.6%)	11(91.6%)
Total	200	46(23.0%)	45 (97.2%)	36(78.2%)

The presence of microorganisms in all types of meat samples validates the earlier findings that the bacteria are widespread in nature including the intestinal tract of humans and animals, which makes the presence of pathogen in different foods inevitable, if the processing of meat is improper. Previous examinations also reported the occurrence of *C. perfringens* in various domestic animals viz. 30% to 60% in beef meat, 30% to 50% in pork and 40% to 80% in poultry (Ryan and Ray, 2004).

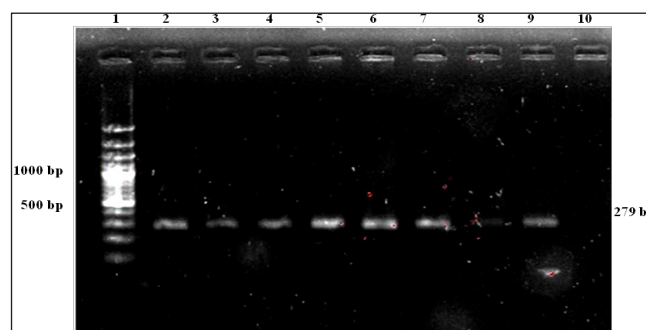


Fig. 1: Agarose gel electrophoresis showing amplified product (279 bp) of rRNA gene of *C. perfringens* isolates. **Lane 1:** DNA ladder; **Lane 2-9:** Amplified product of 16S rRNA gene; **Lane 10:** Negative control

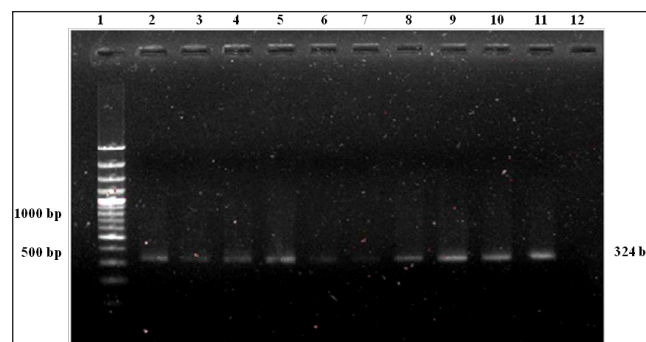


Fig. 2: Agarose gel electrophoresis showing amplified product (324 bp) of lecithinase (*cpa*) of *C. perfringens* isolates. **Lane 1:** DNA ladder; **Lane 2-11:** Amplified product of *cpa* gene; **Lane 12:** Negative control



High presence have also been reported by various workers in our country viz. Singh *et al.* (2006) during examination of 211 meat samples in Bareilly, goat (91.40%), poultry (70.40%) and buffalo (65.70%) had *C. perfringens*. Similarly, in another study, Singh (2010) examined 461 different foods samples and reported 57.70% of *C. perfringens* with poultry (94.41%), goat (58.00%) and fried chicken (16.66%) among meat sample. An investigation in Tamil Nadu from intestinal contents of 267 fresh water fishes revealed the prevalence of *C. perfringens* type A in 49 (18.35%) samples (Das and Jain, 2012). Likewise, examination of 400 food samples in Guwahati, displayed 33 food samples were positive for *C. perfringens* (Gurmu *et al.*, 2013). A high occurrence of the pathogen (81.69%) was reported from 71 chicken meat samples of Bangalore, Karnataka by Prabhu *et al.* (2013). Yadav *et al.* (2016) examined 102 samples of fish and their products in Kolkata and found 24 (23.52%) samples to be *C. perfringens* positive.

Similarly, an observation in abroad (Japan) revealed that out of 200 retail meat samples tested, 71% were found to be contaminated with *C. perfringens* with 33.30% in lamb and 100.0% in ground chicken and duck (Yasuhiro *et al.*, 2008). Likewise, the screening of 200 meat product samples from supermarket and retail meat shops revealed 48.80% and 21.30% *C. perfringens* from ready to cook and ready to eat meat products, respectively (Elham and Nahla, 2011). Another examination revealed 2 and 1.42% *C. perfringens* from imported frozen chicken and imported frozen meat respectively, in Cairo and Giza cities (Elnawawi *et al.*, 2012). A prevalence study (48.27%) was done by Mehtaz *et al.* (2013) in apparently healthy, clinically affected animals, meat and milk. An investigation in Lahore (Pakistan) revealed low prevalence of *C. perfringens* in chicken - 6% (6 / 100), mutton - 5% (5/ 100) and beef - 1% (1 / 100) (Khan *et al.*, 2014). During a study of 90 samples (45 heart - livers and 45 gizzards), Hanifehnezhad *et al.* (2015) observed 46.70% of heart - liver and 17.70% gizzard were contaminated with *C. perfringens*. In another study in Zaria, Tizhe *et al.* (2015) found 14 (3.50%) samples positive for *C. perfringens* out of 400 samples examined from seven different retail markets and abattoir.

The different studies carried out by various workers in our country and abroad showed the presence of *C. perfringens* in meat, indicated the poor hygienic conditions prevailing

in the retail shops, abattoir, etc. The difference in study may be attributed to isolation protocols practiced in laboratories viz. pre-enrichment or enrichment that enhances the recovery of pathogens due to favorable conditions which in turn increases the growth and multiplication and thus, increases the occurrence (Ananthnarayan and Paniker, 2013). The use of antibiotics in selective media, besides decreasing the growth of other bacteria also inhibits the recovery of many strains of desired microorganisms and therefore, lowering the incidence of pathogen in different samples (Singh, 2010). The occurrence in present study in different meat sample didn't show wider variation, which revealed similar conditions in retail outlets.

C. perfringens possess an enzyme lecithinase/phospholipase that break down lecithin to insoluble diglyceride and phosphorylcholine, which results in a white opaque zone around colony on egg yolk agar media. This activity is being exhibited by alpha toxin and is produced *in-vitro* by most of the strains of *C. perfringens*. In this study, all the isolates from buffen, chevon and chicken showed lecithinase activity while 11 (91.66%) isolates of fish meat were positive for lecithinase activity. Few strains of *C. perfringens* were unable to display lecithinase activity in the previous reported works (Sjolkvale *et al.*, 1979; Singh *et al.*, 2006; Agarwal *et al.*, 2009; Yadav *et al.*, 2016). The absence of lecithinase activity by *C. perfringens* in presence of an antitoxin is due to neutralization of lecithinase (Nagler's reaction). This phenomenon isn't common in other *Clostridium* spp viz. *C. baratti*, *C. absonum*, *C. bifermentans*, *C. sordelli* and *C. novyi* because these microorganisms showed lecithinase activity in presence of antitoxin (Ananthnarayan and Paniker, 2013).

C. perfringens ferments the lactose resulting in production of lactic acid and gas in iron milk medium (IMM). The acid coagulates the casein in milk resulting in clear watery fluid. The stormy clot formation is due to fracturing of curd into spongy mass which usually rises above medium surface. This is used for presumptive estimation of *C. perfringens*. For this, the bacteria are incubated in IMM at 45 - 46°C for 14 - 18 hrs. At 45 - 46°C temperature, the growth of other bacteria is decreased but the multiplication of *C. perfringens* is increased due to reduction in generation time interval to 6 - 10 min. Further, the selectivity can be enhanced by addition of neomycin, cycloserine, etc. which inhibits the growth of *E. coli* and *Bacillus* (John *et al.*, 1982; Abeyta *et al.*, 1983).

The molecular study is helpful in establishing genotypic and phenotypic co-relation of different virulence marker of the pathogens. The microorganism was identified with the help of 16S rRNA gene, which is commonly used in the identification of various food borne pathogens. All the isolates displayed presence of lecithinase (*cpa*) gene, which is commonly found in *C. perfringens* type A, a common food borne pathogen. One of the isolates from fish genotypically showed lecithinase (*cpa*) gene, but didn't display phenotypic (lecithinase) activity on egg yolk agar. This indicated the lack of expression of lecithinase enzyme phenotypically. None of the samples was observed to have enterotoxin gene at annealing temperature between 50 – 60°C. Earlier works revealed that only 5 – 6% of *C. perfringens* food borne strains had enterotoxin gene, which lead to food poisoning incidences (Uzal *et al.*, 2014; Nashwa *et al.*, 2016). Most of the food poisoning *C. perfringens* strains are heat resistant and in type A category. In concurrence to present results, previous food sample study have also displayed the absence of enterotoxin gene from the isolates of *C. perfringens* and these isolates were reported from food samples and gastroenteritis cases (Yadav *et al.*, 2016).

The observation of the raw meat samples showed the occurrence of *C. perfringens* in all the species, indicating poor implementation of hygienic measures in the retail premises. Therefore, consumption of such meat may cause gastrointestinal infections and thus lead to food poisoning incidence if not cooked and refrigerated properly.

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