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Toxin-producing *Clostridium perfringens* in cooked cereal food in restaurants in Bangladesh

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Abstract

Clostridium perfringens causes food poisoning in humans worldwide and *C. perfringens* beta toxin is associated with improperly heated or reheated cooked food. A study was undertaken to determine the prevalence of beta toxin-producing *C. perfringens* in cooked Plain rice, Pulao, and Biryani in different types of restaurants (general, well furnished) of four districts [Dhaka (north and south city corporation area), Cumilla, Narayangonj, Gazipur)] of Bangladesh. A total of 200 food samples were examined for the presence of *C. perfringens* and its beta toxin. The positive samples were further tested for the CPB gene (236 bp) of beta toxin-producing *C. perfringens* using PCR assay. Three samples had *C. perfringens* (1.5%), in food samples of the restaurants in Dhaka South City Corporation (DSCC). Beta toxin-producing *C. perfringens* was in one sample (0.5%) in a sample of pulao in the same area during the winter. It is suggested that the prevalence of beta toxin-producing *C. perfringens* was low but, further studies are required in other cities in Bangladesh. (*Bang. vet.* 2023. Vol. 40, No. 1 – 2, 8 – 15)

Introduction

Clostridium is a genus of Gram-positive, spore-forming bacteria, which grow under anaerobic conditions. *C. perfringens* inhabit soil, water, and gastrointestinal tract of various animals and humans (Jelen, 2007). It can produce more than 15 different toxins with different modes of action. Five types of this bacterium are labelled A to E based on their ability to produce single or combination of toxins designated α, β, ε, and ι (Grass *et al.*, 2013). Beta toxin is a major lethal toxin produced by type B and C *C. perfringens* and is a single-chain polypeptide that is more sensitive to trypsin or protease (Chalmers *et al.*, 2008). It plays a major role in necrotic enteritis (food poisoning) in humans and animals (Xiu *et al.*, 2020). In humans, the clinical signs are vomiting, abdominal pain, abdominal cramps, tenesmus, and bloody diarrhoea beginning within 24 hours and lasting less than 24 hours (Nyrah *et al.*, 2017; Shelke *et al.*, 2018: Samul *et al.*, 2013).

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C. perfringens can be transferred to food by soil, dust, or water, due to unhygienic practices. It grows rapidly in food: the vegetative cells may double within a few minutes (Mustafina *et al.,* 2015). The vegetative cells of *Clostridia* are destroyed in a short time above 70°C. However, the Clostridial spore requires temperatures above 121°C for a long time to get destroyed (Zhang *et al.,* 2018).

When the intestinal microbial balance is disturbed by sudden diet change, antibiotic therapy, infection, or enzymatic reaction, this bacterium causes enteritis in a wide range of animals including human beings (Algammal *et al.,* 2015; Zhang *et al.,* 2018). It can cause histotoxic infections in humans like gas gangrene in contaminated wounds, gastroenteritis in humans or animals, necrotic enteritis, and enterocolitis in infants (Alam *et al.,* 2020).

Clostridia and the *spores* are resistant to radiation, which is a serious hazard to health due to the use of radiation in the food industry worldwide (Jelen, 2007). The occurrence of this microorganism, spore, or toxin is an indicator of unhygienic aspects of food products, and environmental contamination (Mehtaz *et al.,* 2013). There is less research on beta toxin or type B *Clostridia* than other bacteria. Epidemiological investigation of food poisoning caused by beta toxin has not been conducted in Dhaka city and the surrounding area. The aim was to detect the prevalence of beta toxin-producing *C. perfringens* in cooked cereal food in selected cities of Bangladesh with an assessment of the risk factors.

Material and Methods

Study area and period: The plain rice, pulao, and biryani were collected from randomly selected restaurants in Dhaka North City Corporation (DNCC) and Dhaka South City Corporation (DSCC) and neighbouring districts Cumilla, Narayanganj, and Gazipur City Corporation of Bangladesh from August 2022 to April 2023.

Study design and sampling strategy: A longitudinal and cross-sectional study was designed. Multi-stage simple random sampling method was done (Sharmin, 2021). A questionnaire was developed to collect location, date, restaurant types, and types of cooked cereal food. Two types of restaurants (general and well-furnished) were included, and 200 samples (40 from each study site) were collected. Three types of popular cooked cereal foods [plain rice, fried butter rice with various spices (pulao), and pulao rice mixed with beef/mutton (biryani)] were included. The individual sample (200 gm) was collected within three hours after cooking and placed in a sterile zip-lock plastic bag and temporarily kept in a cool box. Reheated or frozen samples were not included.

A. Bacteriological test

i. Cultural properties: The samples were prepared immediately after receiving them at the laboratory for the bacteriological study as described by Ezatkhah *et al.* (2016). Nutrient broth, Blood agar, MacConkey agar, and Triple Sugar Iron (TSI) slant were used for bacterial cultivation. The nutrient broth was kept in a candle jar and incubated at 37°C for 24 hours. The suspected *Clostridium* species were grown in anaerobic conditions (Anju *et al.* (2021). In brief, the turbidity in the nutrient broth indicated the presence of anaerobic bacteria. One loopful of a positive culture from the nutrient broth was streaked on the agar base media and incubated at 37°C for 24 hours anaerobically. The morphological properties of the colonies were recorded. The motility test by hanging drop method was conducted as per standard protocol (Agarwal *et al*., 2009).

ii. Staining properties and microscopic observation: The smear was prepared from suspected bacterial colonies followed by Gram-staining method and examined under a light microscope to observe the nature, shape, size, and other criteria of *Clostridium* species as per standard protocol (Shelke *et al.*, 2018).

B. Biochemical tests

Basic sugar fermentation test, oxidase, catalase, indole, methyl red (MR), and Voges Proskauer test (VP) were conducted as described by Eyre (2009).

C. Molecular test

Total genomic DNA was extracted from pure colonies of the bacteria using the Total DNA Extraction Kit as described by Tresha *et al*. (2021). The size (236 bp of the beta toxin of *C. perfringens*) of the target gene (CPB gene) was amplified and the Polymerase Chain Reaction (PCR) was carried out in a Thermal Cycler (2720 Thermal Cycler; Applied Biosystem). The cycling program in PCR for 236 bp of CPB gene amplification included initial denaturation at 94°C for three minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 45 seconds, extension at 72°C for 45 seconds and final extension at 72°C for 4 minutes (Tresha *et al*., 2021). The forward and reverse primers for this PCR reaction were CPB-F: 5′-ACTATACAGACAGATCATTCAACC-3′ and CPB-R: 5′-TTAGGAGCAGTTA GAACTACAGAC-3′, respectively.

The 1.5% agarose gel (w/v) was prepared using 1 x TAE buffer, and 5 µl of ethidium bromide (0.5 µg/µl) was added for 50 ml of agarose gel based on the manufacturer's guideline (URL: http://www.amresco-inc.com). The PCR amplicons (5 µl) were analysed in the gel and 5 µl of 100 bp sized DNA marker was separated in parallel at an initial voltage of 120 volts for 30 minutes at 90 mA and 20 watts. Bands of all PCR amplicons were visualized and compared with gene markers in a UV light chamber.

D. Data analysis

The data were recorded in an Excel spread sheet and analysed using SPSS software (Version 20.0). The correlation between the risk factors [regions, seasons (Rainy: August – October, Winter: November – January, and Summer: February – April), sample types, and restaurant types] and the occurrence of the bacterial load was analysed. The P-value (≤ 0.05) was considered to be statistically significant using Chi-square test.

Results and Discussion

Cultural properties: Eleven out of 200 samples (5.5%) showed turbidity These suspected positive samples were cultured in different agar-based media for confirmation. Three (1.5%) samples showed the characteristic green colonies in MacConkey agar and grey-white colonies with haemolysis in Blood agar. The suspected samples produced acid in the TSI slant and showed a yellow colour (Fig. 1).

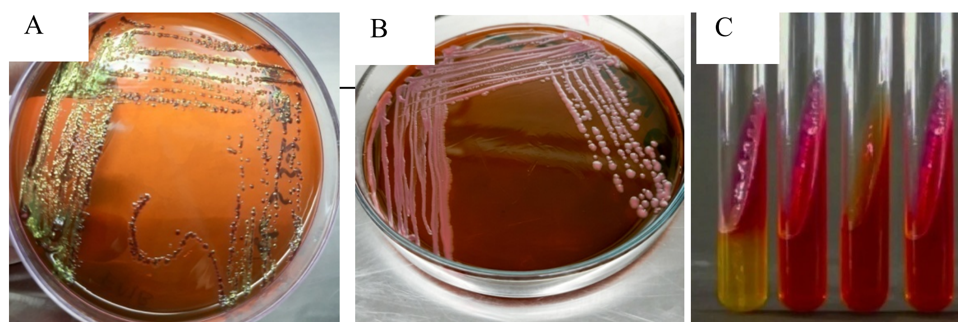

Fig. 1: Green colonies in MacConkey agar (A), grey-white colonies in Blood agar (B) and yellow in TSI slant (C)

Biochemical properties: The biochemical properties of *C. perfringens* are shown in Table 1. These bacteria from suspected three samples fermented the carbohydrates dextrose, lactose, sucrose, fructose, and maltose, and produced acid and gas.

Table 1: Carbohydrate fermentation and other biochemical tests of *C. perfringens*

Serial no.	Name of the tests	Results
1	Carbohydrate fermentation	Acid and gas
2	Indole	Negative
3	Methyl Red	Negative
4	Voges Proskauer	Negative
5	Catalase	Negative
6	Oxidase	Negative
7	Motility	Negative

The cultural, staining, and biochemical properties of *C. perfringens* were consistent with Nyrah *et al.* (2017) and Shelke *et al.* (2018).

Molecular test: Only one sample was positive for beta toxin type of *C. perfringens*. The other two samples did not show the specific band due to primer specification.

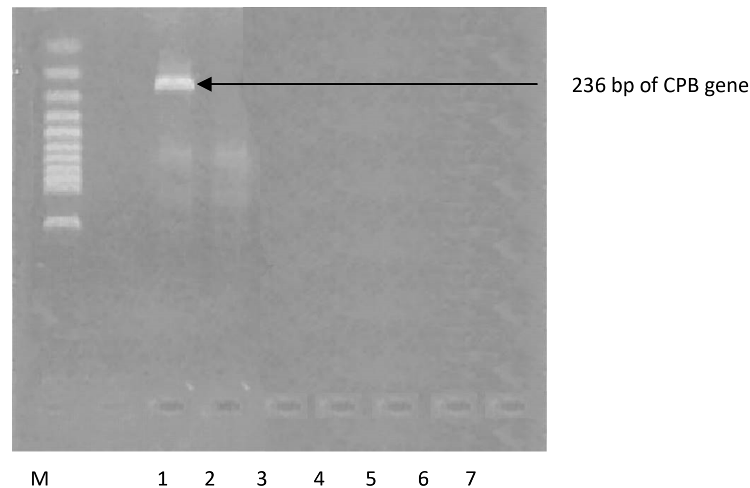

Fig. 2: The 236 bp of the CPB gene of *C. perfringens* (beta toxin) was identified by PCR. Here, M = Marker, 1 = positive sample, 2-7 = negative samples.

The prevalence of beta toxin-producing *C. perfringens* was 0.5% based on molecular identification, but in the bacteriological test, it was 1.5%. Yoo *et al.* (1997) and Tresha *et al.* (2021) detected beta toxin by amplifying the 236 bp of the CPB gene of *C. perfringens*. In DSCC three out of 40 samples were positive (2.5%).

Epidemiological investigation

The one positive case was in a well-furnished restaurant, in Pulao among the cooked cereal foods (Table 2).

Most of the previous works were on the identification of *C. perfringens* type A, whereas a few were on *C. perfringens* type B worldwide. The beta toxin-producing *C. perfringens* had not been detected in Bangladesh. Tresha *et al.* (2021) and Arif *et al.* (2022) found 45 positive cases of *C. perfringens* type A from water and poultry feed samples and found 3.3 % feed samples positive in the summer. There was no positive water sample. The beta toxin-producing *C. perfringens* was detected in 9% of honey samples (Maikanov *et al.*, 2019). Issimov *et al.* (2022) found alpha toxin-producing *C. perfringens* in 27% of raw beef samples in summer. In Egypt, Ghoneim and Hamza (2017) found *C. perfringens* in 2.6%, 5%, and 10% positive cases in a total sample size if 150 in chicken, beef, and sausages, respectively. Bendary *et al.* (2022) in Egypt found *C. perfringens* in 12.6% of beef, 10.6% of chicken, and 10% of raw milk samples. In

Poland, Grenda *et al.* (2017) found beta toxin-producing *C. perfringens* in 24.3% of 260 samples of meat, 4% in ready-to-eat meals, 25% of vegetables and 26% of honey samples. Lower occurrences were recorded here in Bangladesh. It might be due to geographical or environmental variation, the nature of samples, the techniques that were used for the detection of the infectious agents, or hygienic management.

Table 2: The occurrence of beta toxin-producing *C. perfringens*

Risk factors	Group	Total samples	Positive sample	Percentage (%)	p- value
Location	DNCC	40	00		0.403
	DSCC	**40**	**01**	2.5	
	Cumilla	40	00		
	Narayanganj	40	00		
	Gazipur	40	00		
Total sample		**200**	**01**	**0.5**	
Restaurant Types	General	100	00		0.316
	Well-furnished	100	01	1.0	
Total sample		**200**	**01**	**0.5**	
Season	Summer	70	00		0.352
	Winter	65	01	1.53	
	Rainy	65	00		
Total sample		**200**	**01**	**0.5**	
Food types	Plain rice	70	00		0.352
	Pulao	65	01	1.53	
	Biryani	65	00		
Total sample		**200**	**01**	**0.5**	

Legend: DNCC = Dhaka North city corporation; DSCC = Dhaka South city corporation

Conclusion

The 236 bp of the CPB gene of beta toxin-producing *C. perfringens* was detected using PCR techniques in one sample of Pulao rice of a well-furnished restaurant in DSCC during the winter.

Conflict of interests

The authors declare that there is no conflict of interest regarding the study and publication of this work.

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Declaration

The authors declare that the manuscript is original and not previously published or under consideration for publication in any reputed local or international journal.

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