Selecting out of virulent strains of C. perfringens by the preparation process of meat curries possess a public health threat: evidence from a retrospective study

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Original Research

Selecting out of virulent strains of *C. perfringens* by the preparation process of meat curries possess a public health threat: evidence from a retrospective study

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1. Introduction

The member of the gastrointestinal microbiome of humans and life-stock animals, *Clostridium perfringens* (formerly *C. welchii)* is also an opportunistic human and veterinary pathogen causing a spectrum of histotoxic and gastrointestinal diseases namely: gas gangrene, enteritis necroticans, enterotoxaemia, non-foodborne diarrhoea and enterocolitis [1, 2]. Enterotoxigenic strains can produce enterotoxin (CPE) during sporulation in the intestines [3], resulting in characteristic diarrhoea and abdominal cramps when ingested the infective dose ≥ 10^5. More than 20 toxins are identified as the main virulence factors of C*. perfringens* [4]. The production of robust heat-resistant spores under most adversities justifies the ubiquitous nature of this Gram-positive spore bearer in a variety of environments of soils, sewage, dust, food, and feedstuff [3]. Splendid growth of this anaerobe frequently prevails in meat curries due to the availability of at least 12 essential amino acids, optimal pH (6.0– 7.0), and oxidation-reduction potential (around − 200 mV) [5]. This bacterium ranks as one of the commonest three foodborne pathogens in the USA and other developed countries [3, 4, 5]. Eating houses in the main commercial hub-Colombo City facilitate their patronage of 647100 cumulative populations and several hundred thousand floating populations' per day with affordable meat delicacies [4, 5]. There is no information on enterotoxigenic *C.perfringens* food isolates. Thus, the present study objected to comparative analysis of selected virulence factors: heat resistant spore formation, production of *C.perfringens* enterotoxin, heat resistant spore

formation along with *C.perfringens* enterotoxin production of meat curry isolates by the variety obtained from eating houses in the Colombo City.

2. **Materials and Methods**

The sample size was obtained using Lwanga and Lemeshow formula for the descriptive cross-sectional design [6]. The number of eating houses needed from each district to collect samples was obtained proportionate to the number of eating houses in each district as a fraction of the total number of eating houses registered in the Colombo Municipal Council and the total number of samples needed (200). The lottery method was used for selecting eating houses in each sampling area (district) to obtain equal amounts of curry samples from either variety (Chicken 100 and Beef 100), Labelled samples were transported quickly and processed immediately at Food and Water Microbiology Section, Department of Microbiology, Medical Research Institute, Colombo 08. Plate count technique was used to detect this bacterium, [4, 5, 7]. Confirmation of presumptive identification and enumeration performed as described previously [4, 5]. Prepared media were quality controlled using a *C.perfringens* reference strain (ATCC 13124), as the positive control and *Escherichia coli* (ATCC 25922) as the negative control [4, 5]. Uninoculated media were subjected to sterility testing.

Representative subsample (12 chicken and 03 beef) of preserved *C. perfringens* cultures after complete confirming retrieved from the cold room. Stored culture was vortex mixed prior to inoculation. Subsequently, 20ml of freshly steamed thioglycollate medium was inoculated with 2ml of stored culture (preserved in cooked meat medium and they were incubated anaerobically at 37^0C for 24hrs. Sporulation endeavoured using 3 media: Duncan and Strong, Modified Duncan and Strong, and Sporulation Broth. Sporulation endeavoured using 3 media: Duncan and Strong, Modified Duncan and Strong, and Sporulation Broth. Heat resistance was tested at 100^0C for 1 h in distilled water. Finally, the production of enterotoxin was evaluated with a PET-RPLA kit [4, 5]. These data were analysed using SPSS-

21 Statistical Package. Descriptive statistics and Fisher's exact test were used to compare groups ((cell counts <5).

3. Results

The results presented here included 12 chicken and 03 beef curry isolate of *C.perfringens* capable of heat-resistant spore formation, *C. perfringens* enterotoxin production, or both together by the variety of beef curry (Table 01].

Sporulation of *C. perfringens*: The spores stained in pale green with oval central spores central or sub-terminal and distended the sporangium.

Heat resistance of *C. perfringens* spores at 100^{0}C for 1h in distilled water and subsequent germination of spores: The presence of turbidity and Gram-positive rods.

Non-heat resistance of *C. perfringens* spores: The absence of turbidity as well as Gram-positive rods revealed the non-heat-resistant spore formation of this bacterium.

The presence of *C. perfringens* enterotoxin: Agglutination, due to the formation of a lattice structure indicated. Upon settling, a diffuse layer on the base of the well was formed [Figure1].

Absence of *C. perfringens* enterotoxin: Non-formation of a lattice structure i.e. formation of a tight button indicated ***C. perfringens*** enterotoxin being absent or present at a concentration below the assay detection limit (2ng/ml).

Table 01: Selected virulent attributes of *C. perfringens* isolates by the variety of meat curry

Attributes	Chicken n= 12		Beef n=03		p value
	N	%	N	%	
Heat Resistance (HR)	6	50.00	-	-	
C. perfringens Enterotoxin Production	2	16.67	-	-	0.018 (p<0.05)

(CPE)				
Heat Resistance and *C.perfringens* Enterotoxin Production (HR&CPE)	4	33.33	3	100.0
Total	12	(100.0)	3	(100.0)

According to table 01, the differences in HR, CPE and, HR & CPE attributes of curry isolates between chicken and beef were statistically significant ($p<0.05$).

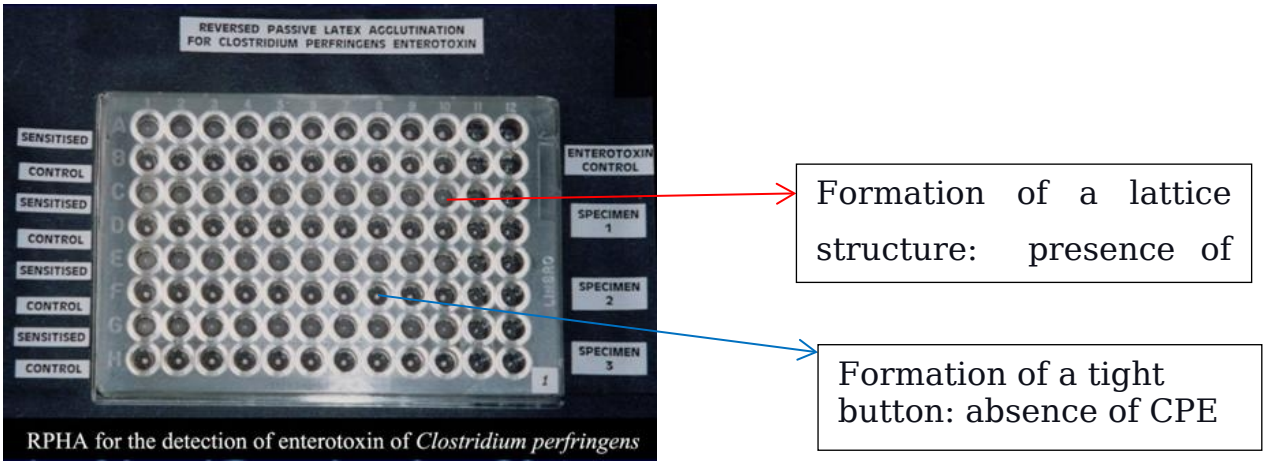

Figure 01: RPLA for the detection of *C. perfringens* enterotoxin

4. Discussion

To the best of the authors' knowledge, this comparative analysis reports the first of its kind conducted in Sri Lanka. Thus, there were no published similar studies elsewhere to compare and contrast the findings. In the present study, 50% of chicken isolates were able to produce heat-resistant spores. Previously, heat-resistant spore formers were detected in raw meat and poultry at 2.2% [5], and cooked meat and poultry at 0.6% [5]. These

values are far below those observed by us. As heat-resistant strains are capable of surviving at cooking temperatures and subsequent germination, these isolates are predominantly hazardous [3, 4, 5] and this notion is confirmed by the finding of epidemic strains of *C. perfringens* were found to be equipped with the highest lethal potency [1]. It has been revealed that approximately 5% of all C*. perfringens* isolates produce a toxin named CPE [8]. However, a nearly threefold increase (16.67%) of CPE-producing chicken isolates was detected in the present study. This far exceeded value can be considered a warning sign as CPE is considered a crucial virulence factor related to the pathology of foodborne illness in humans by this bacterium [9]. The location of the enterotoxin gene (cpe) is either chromosomal or large conjugative plasmids thus, mobilization and spread via horizontal gene transfer is possible [8]. When heat-resistant spore formation is coupled with CPE production it is more hazardous than equipped either with heat resistance or CPE production. Alarmingly, heat resistance with CPE production was observed in 100% and 33.3% of beef and chicken curry isolates respectively. This finding is consistent with the finding from 1998 to 2008, meat, especially beef was incriminated as the most common vehicle of transmission of this bacterium as revealed by food poisoning outbreaks due to *C. perfringens* in the United States [9]. It has been found that an ELISA-based testing approach may be more reliable than RPLA assays for CPE detection [10]. Hence, the usage of PET-RPLA with a sensitivity of 2ng/ml and any level of toxin production has given a negative result been a limitation of the present study. The small sample size (15) of this study is another limitation.

5. Conclusions and recommendations

The higher prevalence of selected virulent factors observed in meat curry isolates demonstrates a high degree of selecting out of virulent strains by the cooking temperature and time of meat curries prepared in eating

houses within Colombo city**.** The subsequent selective proliferation of this bacterium to hazardous levels may impose a public health threat. Heat-resistant spore formation, ***Clostridium perfringens*** enterotoxin production and both together may be significantly affected by the variety of meat curry and this postulation needs confirmation by adequate sample size. Thus, optimized methodologies to increase the sample size of virulent strains are strongly recommended.

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Authors' contributions:

Perera M- assisted all the laboratory experiments, conceptualized, interpreted data, and prepared the manuscript.

Perera I- performed statistical analysis, supported manuscript writing and final editing.

Ranasinghe G- collected & transported samples, performed all laboratory investigations and provided intellectual input in the experimental protocol and manuscript writing.

5. References

1. Grenda T, Jarosz A, Sapała M, Grenda A, Patyra E, Kwiatek K. *Clostridium perfringens*-Opportunistic Foodborne Pathogen, Its Diversity and Epidemiological Significance. Pathogens. 2023 May 26; 12(6):768. doi: 10.3390/pathogens12060768. PMID: 37375458; PMCID: PMC10304509.
2. Uzal, F.A.; Freedman, J.C.; Shrestha, A.; Theoret, J.R.; Garcia, J.; Awad, M.M.; Adams, V.; Moore, R.J.;Rood, J.I.; McClane, B.A. Towards an understanding of the role of *Clostridium perfringens* toxins in human and animal disease. Future Microbiol. 2014, 9, 361–377. [Cross Ref] [PubMed.
3. Perera ML. Sporulation ability of *C. perfringens* isolates from meat curries available in eating houses within Colombo city of Sri Lanka in multiple sporulation media. Research Square; 2022. DOI: 10.21203/rs.3.rs-2287159/v1.
4. Ranasinghe, G., Perera, M.L. and Perera, I., Detection of *Clostridium perfringens* and associated preventive measures to appraise the safe meat curry consumption in the Colombo city. *Journal of Science of the University of Kelaniya Sri Lanka*, 2022,15(1), p.52-66.DOI: https://doi.org/10.4038/josuk.v15i1.8053

5. Ranasinghe, G.R., Occurrence of enterotoxin producing Clostridium perfringens in meat curries available in eating houses within Colombo City 2019-03-27T05:53:26Z http://192.248.21.144/handle/1/1664
6. Lwanga, Stephen Kaggwa, Lemeshow, Stanley & World Health Organization. Sample size determination in health studies: a practical manual/S.K Lwanga and S. Lemeshow. World Health Organization. 1991; https://apps.who.int/iris/handle/10665/40062
7. USFDA Bacteriological Analytical Manual Online 2001http://www.csfan.fda.gov/~ebam (04/05/02)
8. Freedman JC, Shrestha A, McClane BA. Clostridium perfringens Enterotoxin: Action, Genetics, and Translational Applications. Toxins (Basel). 2016 Mar 16; 8(3):73.doi: 10.3390/toxins8030073. PMID: 26999202; PMCID: PMC4810218.
9. Jeong D, Kim DH, Kang IB, Chon JW, Kim H, Om AS, Lee JY, Moon JS, Oh DH, Seo KH. Prevalence and toxin type of *Clostridium perfringens* in beef from four different types of meat markets in Seoul, Korea. Food Sci Biotechnol. 2017 Apr 30; 26(2):545-548. doi: 10.1007/s10068-017-0075-5. PMID: 30263577; PMCID: PMC6049433.March 2022
10. Ishioka T, Aihara Y, Carle Y, Shigemura H, Kubomura A, Motoya T, Nakamoto A, Nakamura A, Fujimoto S, Hirai S, Oishi K, Nagaoka H, Kimura H, Murakami K. Contrasting Results from Two Commercial Kits Testing for the Presence of Clostridium perfringens Enterotoxin in Faeces from Norovirus-Infected Human Patients. Clin Lab. 2020 May 1; 66(5). doi: 10.7754/Clin.Lab.2019.190801. PMID: 32390391.

Figures

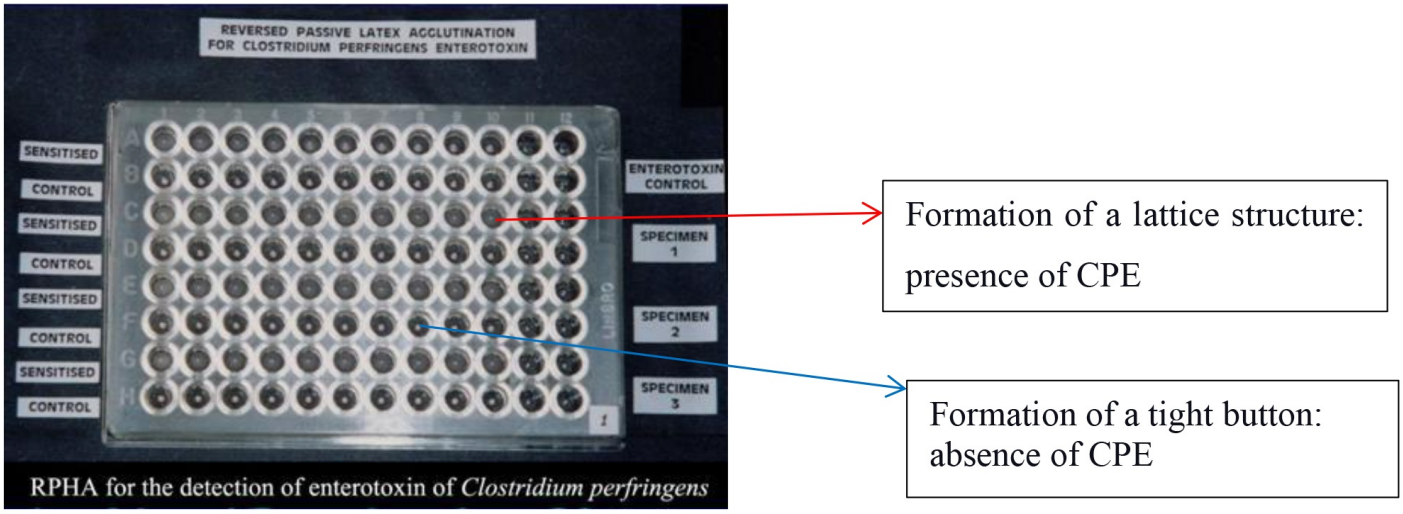

Figure 1

RPLA for the detection of *C. perfringens* enterotoxin