DETECTION OF *Clostridium perfringens* AND ASSOCIATED PREVENTIVE MEASURES TO APPRAISE THE SAFE MEAT CURRY CONSUMPTION IN THE COLOMBO CITY

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ABSTRACT

Sri Lanka is a fabulous tourist destination thus; ensuring food safety has garnered importance in public health. Spicy meat curries are popular among consumers and the majority of them belong to the low-income category. Hence, the aim of this descriptive cross-sectional study was to detect *Clostridium perfringens* and associated preventive measures to appraise the safe meat curry consumption in Colombo city. This spore bearer is ubiquitous in nature and found in a variety of food commodities. Furthermore, this fastidious bacterium is frequently found in meat curries and meat-based dishes. Two hundred meat curry samples comprising 100 chicken and 100 beef were purchased from proportionately selected 200 eating houses in Colombo which have been registered in the Colombo Municipal Council. Spread plate and enrichment techniques were used to optimize the isolation. Subsequently, presumptive identification was done and confirmed by biochemical tests. Confirmed colonies were enumerated and expressed as colony forming units (cfu) per gram of food. Thus, C. *perfringens* was not detected in 61 % of meat curry samples comprising 69 % beef and 53% chicken curries. Safety measures either in preparation and/or storage have been taken by 75- 95% of eating houses, where this cultivable bacterium was not detected. Therefore, as indicated by our findings, safe meat curry consumption in the 21 st century without foodborne pathogens, could not be an over-ambitious goal. Further improvements can be done through the valuable service of Public Health Inspectors (PHIs) about the microbial quality of food.

Keywords: *Clostridium perfringens*, Colombo city, Foodborne pathogen, Meat curries, Tryptose Sulfite Cycloserine.

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INTRODUCTION

Foodborne pathogens are regarded as a global public health threat: consequent to travel, education and trade (Bintsis, 2017). Thus, *Clostridium perfringens* is well established as a common foodborne pathogen in both countries with transitioning and transitioned economies because of the ubiquitous nature of robust spores of this bacterium (Acheson *et al*., 2016, Hailegebreal, 2017). This human pathogen, also known as a veterinary pathogen (Kiu and Hall, 2018), is linked with a spectrum of histotoxic and gastrointestinal (GI) diseases (Sim *et al*., 2015, Heida *et al*., 2016). The magnitude and consequences of foodborne, toxic coinfection by *C. perfringens*, are often undermined due to mildness and self-limitedness (Kiu and Hall, 2018). Meat or poultry containing curries are a frequent accompaniment to the staple diet of rice or bread to titillate the Sri Lankan and tourist palate. This bacterium is a common member of human and animal gut flora, thus contamination of meats via gut microflora (faecal contamination of carcasses) may occur during preparation and storage (Kiu and Hall, 2018, Wijnands *et al*., 2011). Thus, meat consumption in mass gatherings is significantly associated with recent well-documented *C. perfringens* outbreaks (Leung *et al*., 2017, Mellou *et al*., 2019). Colombo city is the densely populated commercial hub of Sri Lanka, where the busy urban lifestyles, predispose to increased patronage of hotels, restaurants and takeaway outlets. With a large number of catering establishments distributed throughout the city to cater to the needs of 647100 cumulative populations (The City Council) and several hundred thousand per day floating population. The eating houses of the city were considered to be ideal for procuring samples. Generally, vegetative cells of *C. perfringens* kill at 70^0C for 5 minutes (Talukdar *et al*., 2017). Moreover, more than 90% of heat-resistant spores of this bacterium are inactivated in boiling water at 90-100^0C for 10-30 minutes (Wang *et al*., 2012). There is also a paucity of information on food safety surveys done for spicy meat curries not only in

Sri Lanka but also in South Asia. Against this situation, the present study was conducted to detect *C. perfringens* and associated preventive measures to ensure safe meat curry consumption in Colombo. It has been hypothesized that the absence of this bacterium in meat curry samples ensures safe meat curry consumption at eating houses in Colombo city.

MATERIALS AND METHODS

In-vitro experiments in the present study pertaining to detection, confirmation and enumeration of *C. perfringens* were conducted in the food and water microbiology laboratory, Medical Research Institute, Colombo 08, Sri Lanka. Associated preventive measures taken against the proliferation of this bacterium in preparation and subsequent storage of meat curries were observed.

Samples/study units of this study: A total of 200 meat curry samples comprising 100 chicken and 100 beef curry portions were collected from 200 eating houses. These eating houses were selected randomly using a lottery method but proportionate to 572 eating houses registered in the Colombo Municipal Council, Public Health Department. Meat curry samples were immediately transported to the food laboratory.

Analytical techniques used for detection, confirmation and enumeration of *C. perfringens*

Preparation of sample dilutions: Each sample was chopped and 25g was weighed on a random basis from different parts of the chopped sample (meat + gravy). It was homogenized for 1-2 min in 225 ml of 0.1% peptone water using a Stomacher 400.A uniform homogenate was obtained with as little aeration as possible. This was the 10^{-1} dilution. Thereafter, 10ml of the initial suspension (10^{-1} dilution) was transferred into a screw-capped bottle containing 90ml of the sterile diluent. This was mixed thoroughly by gently shaking to obtain 10^{-2} dilution. This operation was carried out serially to obtain dilutions up to 10^{-6}.

Spread plate technique: Plating of homogenized food samples was carried out by surface spread method using two, TSC agar [Tryptose Sulfite Cycloserine - Oxoid code: CM 58 with Perfringens (TSC) Selective Supplement B - Oxoid Code: SR 88 and 50% Egg Yolk Emulsion (in-house preparation)], plates from each dilution (SLS 516). Plate count was performed as delineated by SLS 516: part 9: 1986. Plates that contained an estimated 15-150 colonies (SLS 516, USFDA,1978) that were black and surrounded by 2-4 mm opaque white zones were selected. Typical colonies in plates with the highest dilution were counted and 10 colonies from each positive sample were subjected to presumptive identification and confirmation tests outlined below. Based on the results of these tests the final count of *C. perfringens* cells/g was calculated.

Enrichment technique: Bottles (3-4) of prepared CMM (Cooked Meat Medium - Oxoid code: CM 81) were heated at 100^0C for 10min and cooled rapidly without agitation. These bottles were inoculated with 2ml of 1:10 dilution as backup, preceding the plating procedure. These bottles were incubated at 36^0C ± 1^0C for 24-48h. These were discarded if plate counts for viable *C. perfringens* were positive. If not they were plated on well-dried TSC plates and anaerobically incubated at 36^0C ± 1^0C for 24h. Colonies typical of *C. perfringens* were sought and subjected to presumptive identification followed by confirmatory tests.

Presumptive identification: Typical colonies were subjected to presumptive identification by morphological (Gram stain) and proteolytic (Stormy fermentation) tests as described previously (USFDA, 2001).

Confirmation test for *Clostridium perfringens*: Confirmation was performed on presumptively identified colonies by biochemical tests: Motility nitrate (Figure 3) and Lactose gelatin (Figure 4) as described previously (SLS 516, USFDA,1978, USFDA, 2001). All batches of prepared media were quality controlled. Performance testing was

done with a reference strain (ATCC 13124), which was the positive control. *Escherichia coli* (ATCC 25922) was used as the negative control. Uninoculated media were subjected to sterility testing.

Confirmation of spore formation: Spore formation was confirmed by spore staining as described previously (Schaeffer and Fulton, 1933).

Documentation of preventive measures: Cooking temperature, cooking time, storage temperature and storage time of meat curries in warmers and disposable containers were documented.

Statistical analysis: The laboratory data entry and analysis were performed using SPSS-21, Statistical Software Package. Descriptive and inferential statistics were used. Pearson's chi-square test was used to determine asymptomatic and exact significance. The significance level was <0.05 at a 95% confidence level. Descriptive statistics were used to find out percentages of preventive measures.

Interpreting results of *C. perfringens* colony count: Average colony count of the highest dilution that contained 15–150 colonies were taken. e.g. If the average count obtained with 10^{-4} dilution of sample was 85, and 8 of 10 colonies tested were confirmed as *C. perfringens*. The number of *C. perfringens* cells/g of food is

85 x 8/10 x 10,000

680000

6.8 x 10^5 cfu/g of food.

But dilution factor is tenfold higher than the sample dilution because only 0.1 ml was spread on each plate

Hence, the final calculation would be 6.8 x 10^5 x10

6.8 x 10^6 cfu/g of food

RESULTS

1. Presumptive identification of *C. perfringens*) by typical colonies

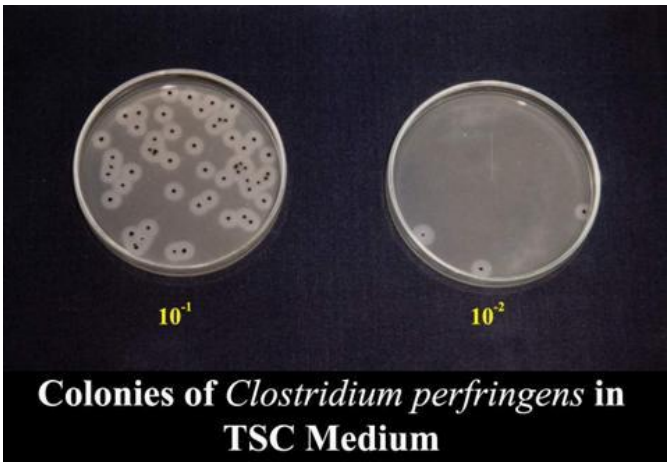

Figure1: Due to lecithinase activity, the typical coloniesfringens were black in colour, situated within the depth of the agar and surrounded by 2-4 mm opaque white zones.

2. Confirmation of spore formation (Figure 2) by spore staining

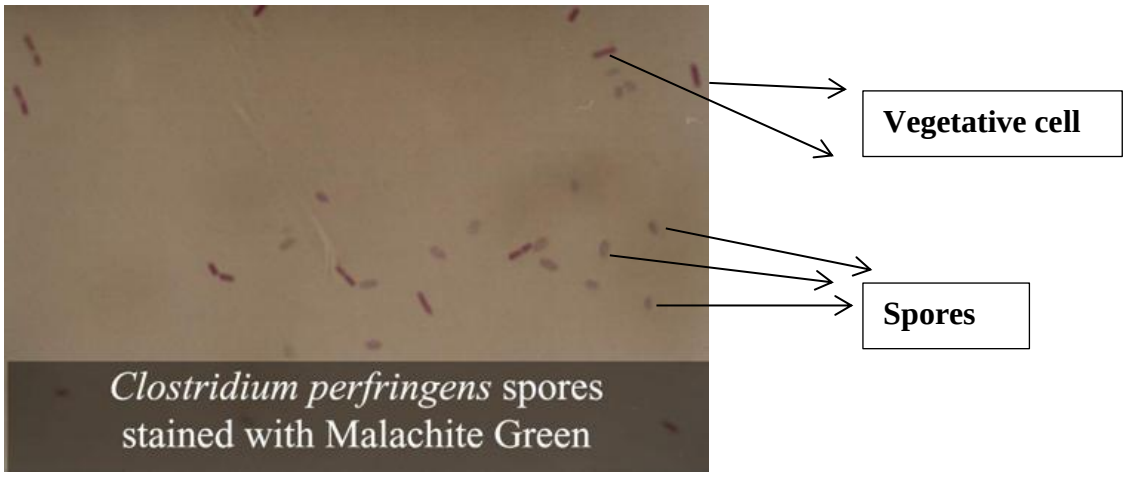

Figure 2: Spore staining - Characteristic appearances of *C. perfringens* vegetative cells were straight rods with blunt ends, stained in red.

3. Motility Nitrate

As shown in figure 3, **the** development of red colour within 5min was a positive result for reducing nitrates to nitrites. No change in colour (absence of red colour) indicated either nitrate was not reduced at all or was entirely reduced to N_2. The development of a red colour after the addition of Zn dust (within10min) was the negative result for the reduction of nitrate.

Figure 3: Mortility Nitrate reaction

4. Lactose gelatin

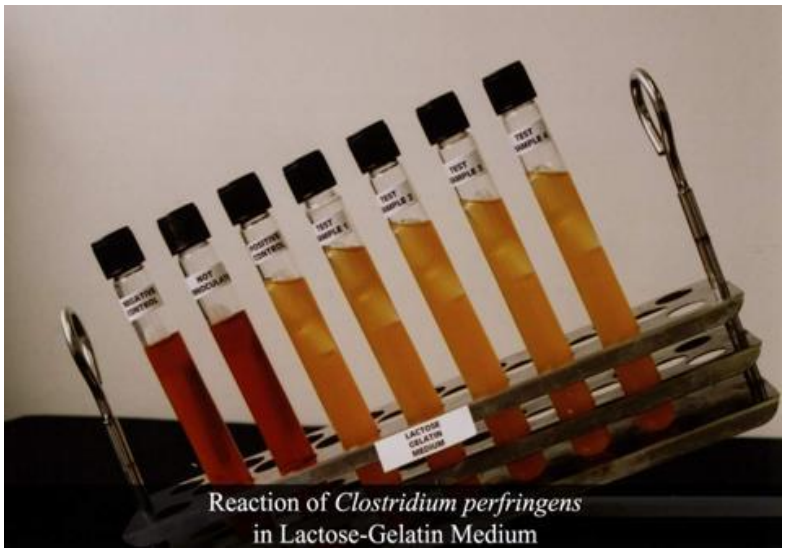

Figure 4: Lactose gelatin reaction

As delineated in figure 4, the colour change from red to yellow and the formation of air bubbles in the Durham tube was the positive result for lactose fermentation. No colour change from red to yellow and non-formation air bubbles was the negative result for lactose fermentation

5. Detection status of *C. perfringens* from meat curries

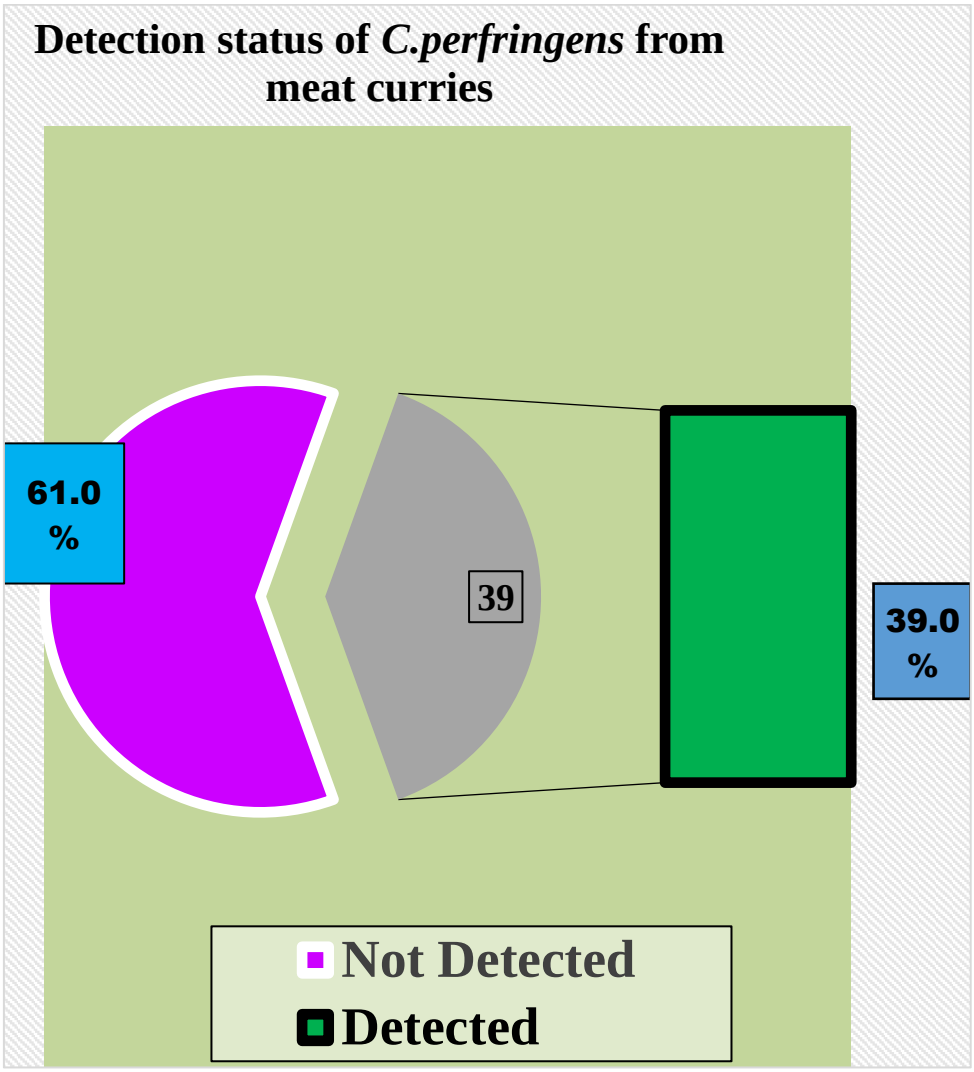

Figure 5: Percentage (%) of detection status of *C. perfringens* from meat curries

As depicted in figure 5, *C. perfringens* was not detected in 61.0 % of samples either by plate count or enrichment technique. In contrast, *C. perfringens* was detected and confirmed by an array of morphological and biochemical tests, in 39.0 % of meat curry samples.

6. The detection of culturable *C. perfringens* by the variety of meat curry

As per Table 1, Chicken curries (47%) (Figure 6) and beef curries (31%) (Figure 7) harboured this bacterium. A statistically significant association ($p< 0.05$) was observed between the detection of *C. perfringens* in two varieties of meat curry, with 95%

confidence limits of (47 ± 9.38)% and (31 ± 9.11)%) for chicken and beef curries respectively.

Table1: Detection of *C. perfringens* by the variety of meat curries

Meat Variety	***Absence of C.perfringens***		***Presence of C.perfringens***		**Total Number**
	No	**%**	**No**	**%**	
Chicken	53	53.0	47	47.0	100
Beef	69	69.0	31	31.0	100
Total	122	61.0	78	39.0	200

$X^2 = 5.380$, df = 1, p = 0.02, p <0.05

Figure 6; Chicken curry

Figure 7; Beef curry

7. **Preventive measures are taken to avoid time-temperature abuse during the preparation and storage of meat curries available for sale in eating houses**

Figure 8: Preventive measures are taken to ensure the safe meat curry consumption in the Colombo city

As shown in Figure 8, Satisfactory cooking temperature was practiced by 75% of eating houses but 65% cooked for a satisfactory time. In the storage, satisfactory temperature and time were observed in 70% and 80% of eating houses respectively.

DISCUSSION

To the author's knowledge this study on the detection of *C.perfringens* and associated preventive measures in food preparation and storage to appraise the safe meat curry consumption in the Colombo city represents the first of its kind conducted in Sri Lanka. Thus, there were no published similar studies elsewhere to compare and contrast the findings. *C.perfringens* was detected in 31% of meat curry samples. When it comes to varieties, 47 % of chicken curries harboured this bacterium whereas, 31% of beef curries contained cultivable *C. perfringens*. Undercooked areas have been observed in chicken pieces due to uneven distribution of heat during cooking, when compared with beef cubes, which may contribute to the statistically significant difference ($p<0.05$) in the detection of *C.perfringens* in chicken curries compared with beef curries. This could be possibly attributed to the larger size of the cooked piece of chicken compared to meat, thereby resulting in indifference in the surface area of the portion to be cooked. This important finding merits further exploration.

In contrast, *C. perfringens* was not detected in 61.0% of meat curry samples to fulfill the standard criteria of 15-150 colonies at least in one dilution ranging from 10^{-1} to 10^{-6}. Hence, preventive measures in food preparation and storage have been taken against the multiplication of this bacteria has been observed in the eating houses, which sold these meat curry samples. It has been observed that 75% of eating houses boiled the curries at 100^0C. However, 65% of eating houses cooked curries for 10-30 minutes (12). These findings are in line with previous laboratory observations. Typical D_{90} values for *C. perfringens* vegetative cells are 0.1 to 0.5 min (6 to 30 S) at 65^0C. When the temperature increases by 5^0C, the death rate increases about tenfold. Hence, it is recommended to cook food until all parts reach at least 70^0C. Furthermore, inactivation of 90% of *C. perfringens* spores (which can be considered heat-sensitive spores) was observed when they were

incubated in water at 90 to 100°C for 10 to 30 minutes in a study conducted by Wang and colleagues in 2012 (12). *Clostridium perfringens* enterotoxin, unlike other extracellular toxins is not secreted from vegetative cells. It is produced only when the organism is sporulating either in vivo or in vitro (7). The enterotoxin is heat labile, with heating in saline at 60^0C for 5 min destroying its biological activity (7). Thus, the Sri Lankan way of meat curry preparation is safer when considering heat sensitive nature of vegetative cells, heat-sensitive spores and the heat labile nature of *C. perfringens* enterotoxin.

When contaminated food with large numbers of vegetative cells is consumed, cells sporulate in the intestine producing enterotoxin, which results in symptoms of gastroenteritis. Hence, appraisal of storage practices is of utmost importance to mitigate germination of survived heat-resistant spores and the proliferation of vegetative cells obtained by cross-contamination during storage. In storage, 70% of eating houses' storage temperature was satisfactory as they stored the samples in warmers (50 -60^0C). This prevents not only the germination of heat-resistant vegetative cells but subsequently proliferation as well of those into the hazardous level of ≥ 105 cfu/g of food in storage with the shortest generation time of 7 minutes. In contrast, storage time was satisfactory in 80% of eating houses as they were stored either in warmers or at room temperature with a relatively rapid turnover. In eating houses, regular customers purchased the meat curry portions within 1-2 hrs of preparation even stored at room temperature in metal containers. Hence, there was no suitable temperature or adequate time for the germination and proliferation of heat-resistant spored survived in cooking procedures in 70% and 80% of eating houses respectively.

CONCLUSIONS

C. perfringens has not detected in 61 % of meat curry samples consisting of 53% of chicken and 69% of meat curries. Safety measures were practiced in 65 % - 80 % of eating

houses to prevent the propagation of this bacterium during preparation and storage in meat curries where this bacterium was not detected.

RECOMMENDATIONS

Safe meat curry consumption for the 21st century without foodborne pathogens is possible. This scientific evidence should be transformed into meticulous compliance with food safety measures by owners, employees and consumers of food outlets. The services of PHIs who render their services on food safety are strongly recommended to improve the microbial quality of ready-to-eat food available for sale in food establishments of the Colombo city.

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