Sporulation ability of *C. perfringens* isolates from meat curries available in eating houses within Colombo city of Sri Lanka in multiple sporulation media

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Research Article

**Keywords:** Clostridium perfringens, Sporulation, Modified Duncan and Strong, Sporulation Broth, Duncan and Strong, Strain Specificity

**Posted Date:** November 18th, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-2287159/v1

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Abstract

*Clostridium perfringens* is a well-known foodborne pathogen across the globe. Sporulation is of utmost importance in the growth curve of this anaerobic bacterium to withstand extreme environmental conditions and deprivation of nutrients. Present study was conducted to sporulate 78 of *C. perfringens* isolates obtained from meat curries available in eating houses within Colombo City, Sri Lanka using three sporulation media namely: Modified Duncan and Strong (MDS), Sporulation Broth (SB) and Duncan and Strong (DS). Other components were more or less same in these sporulation media but the slowly fermentable carbohydrate source was different in each media. Raffinose (MDS) was superior to soluble starch (SB) and starch (DS) stimulating sporulation of meat isolates. Modified Duncan and Strong medium was the most efficient of the three sporulation media with total of 41 meat isolates were sporulated when compared with 28 and 26 in SB and DS respectively. Sporulation was higher for chicken isolates 33(70.2%) than for beef isolates 15(48.4%). There was a statistically significant difference in sporulation of *C.perfringens* isolates from both chicken and beef curry isolates, in MDS when compared with DS and SB individually as well as combinations MDS vs. DS and MDS vs. SB  (p<0.05). This study highlights the importance of optimizing these three media and usage of additional media to sporulate non sporulate *C. perfringens* isolates.

1. Introduction

*Clostridium perfringens* is a Gram positive, spore forming, rod shaped bacterium (Abela-Ridder et al. 2013; Kuchenmüller et al., 2013; Ma et al., 2012). This ubiquitous bacterium (Hassan et al. 2015) can be found in soil, sewage, dust, polluted water, faeces of animals and humans, animal origin of food as well as feed stuffs (Aceson Petal, 2016; Organization and Aquaculture 1999). Chicken and beef gravies are reported as most commonly incriminated food of *C.perfringens*, food poisoning outbreaks (Kuchenmüller et al., 2013). Meat and meat based food are enriched with at least essential amino acids (12), optimal pH (6.0–7.0) and Oxidation Reduction Potential (around −200 mV) for the luxurious growth of this fastidious anaerobe (Doyle, E 2002; Fazil et al. 2002). *C. perfringens* is well documented as a human and veterinary pathogen across the globe. ( Kiu and Hall 2018; Sim et al., 2015 ). The *C. perfringens* enterotoxin (CPE) is responsible for causing the gastrointestinal symptoms. Alarmingly, foodborne illness (Acheson et al., 2016; Hailegebreal et al., 2017; Heida et al., 2016) caused by this bacterium ranks as the second most prevalent bacterial food-borne illness in the USA, with approximately 1 million cases/year (Li et al. 2016). This spore bearer possess the ability to sense the “environmental stress” and undergo morphological changes, in order to produce metabolically dormant, stress resistant endospores (Huang et al., 2007), which facilitate survival in extreme or nutrient-deficient conditions (Kiu and Hall 2018). Hence, increased resistance to food environment stresses including: heat, cold, UV radiation, desiccation and preservatives has been demonstrated by the spores of food poisoning strains of *C. perfringens* (Li et al. 2016). It is essential to sporulate *C. perfringens* food isolates in laboratory media to detect the ability to form enterotoxin (De Jong et al., 2002). Thus, several laboratory media have been formulated to enhance the sporulation of *C. perfringens* by in-cooperating a non-fermentable carbohydrate source, either starch or
raffinose to create nutrient deficient environment, but none stimulates sporulation for all strains. Thus, sporulation of *C. perfringens* is strain and medium dependent. Against this challenging backdrop, this study was aimed at assessing in-vitro sporulation of 78, *C. perfringens* isolates from meat curries available in eating houses within Colombo city, Sri Lanka in three laboratory media, namely; Duncan & Strong (DS), modified Duncan & Strong (MDS) and sporulation broth (SB).

2. Materials And Methods

In order to stimulate the sporulation capacity of the 78 *C. perfringens* meat isolates, 3 sporulation media were used as follows:

2.1 Preparation of Sporulation Media

2.1.1 *Duncan and Strong (DS) Sporulation Medium* (Labbe and Duncan, 1975; Labbe, Somers and Duncan, 1976).

The formula of the medium is as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity for 100ml*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone</td>
<td>1.5g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.4g</td>
</tr>
<tr>
<td>Starch</td>
<td>0.4g</td>
</tr>
<tr>
<td>Sodium thioglycollate</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.7H$_2$O</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100ml</td>
</tr>
</tbody>
</table>

* The ingredients were dissolved in the 100ml of distilled water, and pH was adjusted to 7.2. Then 15 ml portions were dispensed into individual screw scrapped universal containers and sterilized by autoclaving at 121°C for 15 min.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity for 1000ml*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone</td>
<td>15g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>4g</td>
</tr>
<tr>
<td>Raffinose</td>
<td>4g</td>
</tr>
<tr>
<td>Sodium thioglycollate</td>
<td>1g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$.7H$_2$O</td>
<td>10 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

* These ingredients were dissolved in 1000.0 ml of water. Then pH was adjusted to 7.8 ± 0.1, using filter-sterilized 0.66 M sodium carbonate. Then 15 ml portions were dispensed into separate screw capped bottles and autoclaved for 15 min at 121ºC.

### 2.1.3 Sporulation Broth (USFDA, Bacteriological Analytical Manual Online, 2001).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity for 1000ml*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polypeptone</td>
<td>15 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3 g</td>
</tr>
<tr>
<td>Starch soluble</td>
<td>3 g</td>
</tr>
<tr>
<td>MgSO$_4$ (anhydrous)</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Sodium thioglycollate</td>
<td>1 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>11 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

* Ingredients were dissolved in 1000ml of d.H$_2$O. The pH was adjusted to 7.8 ± 0.1. Then 15 ml portions were dispensed into individual screw cap bottles. Finally autoclaved for 15 min at 121ºC.

### 2.2 Preparation of inoculum

20ml of freshly steamed thioglycollate medium was inoculated with 2ml of stored *C. perfringens* culture (preserved in cooked meat medium). Stored culture was vortex mixed prior to inoculation. Three thioglycollate bottles were inoculated with each stored culture (USFDA, Bacteriological Analytical Manual Online, 2001).

### 2.3 Inoculation of Sporulation Media
Then 20ml of each sporulation medium was inoculated with 0.2 ml of 6–8 h C. perfringens culture. All 78 C. perfringens cultures were inoculated into 3 sporulation media i.e. Duncan & Strong, Modified Duncan & Strong and Sporulation Broth. They were incubated at 37°C anaerobically for 24 hours (USFDA, Bacteriological Analytical Manual Online, 2001).

### 2.4 Preparation of Smears for Spore Staining

Then bottles were centrifuged at 4500 R.P.M. for 10min. A deposit could be observed in sporulated cultures (Fig. 1, Page 04). Excess supernatant was discarded, retaining only 3-5ml with the sediment. Sediments were resuspended and smears were prepared on slides.

### 2.5 Confirmation of Spore Formation

Spore formation was confirmed by spore staining (Schaeffer & Fulton, 1933) and direct microscopy as described previously.

### 2.6 Data Analysis

Data were entered and analysed using SPSS-21 Statistical Package. Descriptive and inferential statistics were used. Pearson’s chi square test, continuity correction, likelihood ratio, Fisher’s exact test and linear-by-linear association were calculated. Asymptotic and exact significance were determined. The level of significance was < 0.05 at 95% level of confidence.

### 3. Results

The results of sporulation status of C. perfringens 78 meat curry (47 Chicken and 31 Beef) isolates were presented here. Comparisons were made to highlight the efficiency of sporulation media either individually or in combinations.

#### 3.1 Sporulating media

In the absence of fermentable carbohydrate source C. perfringens tends to form dormant spores,

#### 3.2 Confirmation of sporulation by Spore Staining (Schaeffer & Fulton, 1933)

#### 3.1 Ability of sporulation of C. perfringens in laboratory media

Figure 3 depicts the ability of sporulation of 78 C. perfringens meat curry isolates in sporulation media. Of them 48 isolates were able to sporulate, whereas 30 were not sporulated.

#### 3.2 Performance of three sporulation media

Figure 4 delineates the performance of sporulation media. Of them, Modified Duncan and Strong appeared the best (41 isolates), followed by sporulation broth (28 isolates) and Duncan and Strong
3.2 Sporulation/non sporulation of C.perfringens isolates in laboratory media by variety of meat curries

According to Fig. 5, sporulation was higher for chicken isolates 33(70.2%) than for beef isolates 15(48.4%). In contrast, more beef isolates 16 (51.6%) did not sporulate compared with chicken isolates 14 (29.8%).

3.3 Sporulation of the C. perfringens isolates from meat curries by individual media

Table 1: Sporulation of C.perfringens isolates (from Chicken and Beef) in individual DS, MDS and SB media

<table>
<thead>
<tr>
<th>Variable</th>
<th>Chicken (n=09)</th>
<th>Beef (n=08)</th>
<th>value</th>
<th>significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sporulation media</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>individually</td>
<td>N %</td>
<td>N %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS only</td>
<td>- (0.0)</td>
<td>- (0.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDS only</td>
<td>7 (77.8)</td>
<td>5 (62.5)</td>
<td>0.047*</td>
<td>(p&lt;0.05)</td>
</tr>
<tr>
<td>SB only</td>
<td>2 (22.2)</td>
<td>2 (37.5)</td>
<td>0.131</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>09 (100.0)</strong></td>
<td><strong>08 (100.0)</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Fisher’s exact test to compare groups (cell counts < 5)

Table 1 shows the individual performance of 3 sporulation media. There was a statistically significant (p < 0.05) difference in sporulation of C.perfringens isolates from both chicken and beef curry isolates, in MDS when compared with DS and SB individually.

3.4 Sporulation of the C. perfringens isolates from (Chicken and Beef) curries by combination of sporulation media

Table 2: Sporulation of the C. perfringens isolates of chicken and meat curries by combination of 3 sporulation media
Table 2 presents the successful combinations of sporulation media. From both varieties of curries (Chicken and Beef) statistically significant associations were observed between MDS vs. DS and MDS vs. SB (p = < 0.0001 and p = 0.003 respectively).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Chicken (n=24)</th>
<th>Beef (n=08)</th>
<th>p-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporulation media</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>individually</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS &amp; MDS</td>
<td>6 (25.00)</td>
<td>2 (25.0)</td>
<td>0.0001*</td>
<td>(p&lt;0.05)</td>
</tr>
<tr>
<td>DS &amp; SB</td>
<td>2 (8.33)</td>
<td>1 (12.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDS &amp; SB</td>
<td>5 (20.83)</td>
<td>1 (12.5)</td>
<td>0.0003*</td>
<td>(p&lt;0.05)</td>
</tr>
<tr>
<td>DS, MDS &amp; SB</td>
<td>11 (45.84)</td>
<td>4 (50.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>09 (100.0)</td>
<td>08 (100.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Fisher’s exact test to compare groups (cell counts <5)

4. Discussion

There is a paucity of information in similar studies to compare the results of the present study, though enterotoxin production is sporulation specific. Moreover, sporulation of *C. perfringens* is extremely poor in invitro compared with invivo (De Jong et al., 2002, Hsieh and Labbe, 2007). Handful of laboratory media has been developed to maximize invitro sporulation of this bacterium. Thus, sporulation of broad spectrum of *C. perfringens* strains seemed dependent on sporulation promoting compounds which may facilitate the resting stage than the active vegetative stage (De Jong et al., 2002; Hsieh and Labbe, 2007). Favourable pH and inoculation levels are also equally important in this regard (De Jong et al., 2002). Amino acids, minerals and a slowly fermentable carbohydrate as a source of carbon (C) are the basic ingredients in any sporulation medium (Meyer and Tholazan., 1999; De Jong et al., 2002). In the present study raffinose, starch and soluble starch were in cooperated as carbohydrate source in MDS, SB and DS respectively. None of the sporulation media were contained easily fermentable glucose as the carbohydrate source. This finding is on par with previous findings and recommendations (De Jong et al., 2002; Hsieh and Labbe, 2007). Raffinose was identified as the most efficient slowly fermentable carbohydrate source superior (Hsieh and Labbe, 2007) not only to soluble starch but insoluble starch also. Hence, modified Duncan and Strong medium (MDS) with raffinose resulted in a considerably higher proportion of sporulating isolates, which was statistically significant when compared with conventional Duncan and Strong medium which did not contain raffinose. Furthermore, soluble starch in DS medium was better than insoluble starch in SB medium. Similar findings were obtained in the same study mentioned earlier (De Jong et al., 2002; Hsieh and Labbe, 2007). However, De Jong and colleagues also found that some strains of *C. perfringens* sporulated poorly in the presence of starch, whereas with other strains sporulation was promoted (De Jong et al., 2002). Strain specificity in sporulation was reported as an inherent limitation in previous studies (De Jong et al., 2002), thus inability of sporulating of 38.46% of...
C. perfringens meat curry isolates (29.79% of chicken and 51.61 of beef) mainly due to strain specify, rather effectiveness of slowly fermentable carbohydrate source.

Rapidly metabolizable carbohydrates such as glucose and maltose are vigorously fermented by vegetative cells to produce acid with consequent lowering of pH. Hence, rapidly metabolizable carbohydrates repress the sporulation process by enhancing the active vegetative growth. The pH range (pH 6.0 to 8.0) for sporulation is narrower than for growth. Most laboratory media for sporulation specify an initial pH of 7.5 or higher (Labbe 1989). This pH was ideal for sporulation of many strains but not for all (De Jong et al., 2002). This seems one of the reasons for least performance of DS compared with other two media.

It is reasonable to assume that considerable number of heat resistant spores which survived during cooking of meat curries did not sporulate in any sporulation media. This means sporulation media may not have been optimal.

It is therefore concluded that for induction of sporulation in laboratory conditions is a cumbersome task which essentially need more than one medium. Also based on the evidence of the present study the superiority of raffinose containing media in comparison to starch containing media is declared. Although these media are especially designed to support sporulation of strains isolated from foods and stools of patients involved in food poisoning, several strains of C. perfringens still do not sporulate in any laboratory media. Addition of theophylline to DS medium was shown improved results of sporulation (De Jong et al., 2002). Moreover, another sporulation medium: peptone bile theophylline starch medium was appeared as the most promising sporulation medium tested as peptone bile theophylline starch medium yielded highest spore numbers (2.5 3 10^5 /ml) (De Jong et al., 2002). Hence, inability of including afore mentioned medium in the present study or at least improving DS medium by addition of theophylline and controlling inoculum levels preparing the inoculum (overnight or log – phase culture) and the amount of inoculum (1% or 5%), were the other limitation of the present study.

Hence further improvements to overcome the limitations stated here are highly recommended to optimize sporulation of strains of C. perfringens in laboratory media.

Declarations

Conflict of interest and funding

The authors declare no potential conflicts of interest with respect to authorship and/publication of this article.

Funding Agent

This study was funded by Medical Research Institute, Colombo 08, Sri Lanka under the project No 02/2002.
References


**Figures**

![Figure 1](image1)

**Figure 1**

Growth of *C. perfringens* in Sporulation media
Figure 2

Spore Staining

Sporulation of meat curry isolates of *C. perfringens*

- Sporulated: 48
- Non-sporulated: 30

Figure 3

Sporulation of *C. perfringens* isolates from meat curry samples

The spores stained pale green and were oval, central or sub terminal and distended the sporangium.
Figure 4

Performance of Sporulation media

Figure 5

Sporulation status of *C. perfringens* by the variety of meat curry