Heat Resistance and α-Toxigenicity of Clostridium perfringens Strains in Normal Intestines of Japanese

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ABSTRACT

Heat-resistant strains of Clostridium perfringens were isolated from normal feces samples of Japanese people more frequently than from European people. However, the predominant number of the C. perfringens cells in the human intestines were sensitive to the heat at 100°C for 10 min. This proved to be true of the feces samples which were demonstrated to carry the organism resistant to 100°C for 60 min. Further studies indicated that the more the number of the organism in the intestines, the higher was the recovery ratio of the heat-resistant strains. Concomitant estimation for α-toxigenicity of those strains isolated from unheated feces samples revealed that the α-toxigenicity ranged mainly between 0.05 and 0.9 α-antitoxin equivalents.

In Clostridium perfringens food poisoning, Hobbs et al. [6] demonstrated that the organisms could be isolated from approximately 90% of feces samples heated at 100°C for 60 min. On the contrary, organisms were isolated in only about 5% of normal feces samples treated under the same conditions. Further investigations performed in Europe confirmed their finding [4, 7, 9, 15]. Turner [15], however, isolated the heat-resistant strains in 63% of fecal samples collected from Chinese at Hong Kong who were not affected with C. perfringens food poisoning. Several investigators in Japan also found that healthy Japanese carried the heat-resistant strains in high percentages [1, 2, 8, 10]. During our surveys for a correlation between heat-resistance and α-toxigenicity of C. perfringens in human feces, we encountered a finding that the more the total number in feces, the higher was the recovery ratio of the heat-resistant cells. Considering the high recovery ratios in Japan and its significance in the food poisoning, we undertook to analyze the correlation between the two phenomena mentioned above. Also, α-toxigenicity of C. perfringens strains most prevalent in Japanese intestines was estimated.

MATERIALS AND METHODS

Isolation of heat-resistant strains. Feces samples about the size of pea were suspended in tubes of 1% lactose cooked meat broth and immediately heated at various temperatures for different lengths of time. The heated suspensions, after cooling were
incubated overnight. One loopful of each culture was plated on a Zeissler's plate and colonies resembling those of *C. perfringens* were successively subcultured on the same medium to make sure of the purity. Identification was performed by use of the method of Willis and Hobbs [16].

**Isolation of *C. perfringens* strains of normal flora in human intestines.** One loopful of each feces samples was directly smeared on Zeissler's plate containing kanamycin (100 μg/ml). Strains established from the developed colonies were purified and identified as mentioned above. These were regarded as strains of *C. perfringens* most commonly prevailing in the human intestines and used for the examinations for the heat resistance and α-toxigenicity.

**Quantitative estimation of *C. perfringens* cells in human feces samples.** One ml portion of each feces samples was suspended in a 9-ml portion of 0.85% NaCl solution. Serial ten-fold dilutions were prepared. One-tenth ml of the diluted suspensions were plated on Zeissler's plates, and colony counts performed.

**Estimation of α-toxin, the medium used for α-toxin production and heat resistance test.** These were carried out as previously reported [12, 17]. Toxic strength is shown by α-antitoxin equivalents (AE).

**RESULTS**

**Heat-Resistance of *C. perfringens* in Human Intestines**

Of a total of 70 feces samples which were heated first, positive growth of *C. perfringens* was observed in 27 (39%). In the course of this study, 20 of the 27 feces samples were directly smeared on Zeissler's plate agars containing kanamycin and strains of *C. perfringens* were isolated from colonies developing on the plates. All of these isolates, when cultured in cooked meat broth for 48 hr and subjected to the heat-resistance test at 100°C for 10 min, proved to be heat-sensitive. This finding suggested that the heat-resistant cells of *C. perfringens* in each feces sample were present in small numbers. In a further experiment to confirm this finding, a loopful of 46 feces samples were directly smeared on the kanamycin Zeissler's plates and 5 to 10 substrains were isolated from colonies developing on each plate. Consequently, a total of 306 *C. perfringens* strains were established. Also, the remaining portions of feces in a size of pea were suspended in 1% lactose cooked meat broth, immediately heated at 100°C for 60 min and then incubated. Positive growth of the organism could be obtained in 20 (45%) of the 46 samples examined. Of the 306 strains mentioned above, 133 strains obtained from the 20 cases which had given rise to heat-resistant cells were subjected to the heat-resistance test at 100°C for 10 min as mentioned already. Only but 5 strains were heat-resistant.

**Number of *C. perfringens* in Human Intestines**

In the course of the above-mentioned experiment we noted that the more the colonies developing on the plates directly smeared by feces samples, the higher was the ratio of *C. perfringens* strains recovered from the heating at 100°C for 60 min. We, therefore, undertook a further experiment to analyze a possible correlation between the total number in feces samples and the recovery ratios of the heat-resistant strains, and 57 feces samples were employed. The results are shown in Table 1, and confirm a possible validity of the assumption mentioned above.

Furthermore, based on reports that old people carried the heat-resistant strains more frequently than younger [6, 11], the correlation between the total number of
C. PERFRINGENS IN NORMAL HUMAN INTESTINES

Table 1. Number of C. perfringens in normal human intestines and recovery ratio of heat-resistant cells from the feces samples

<table>
<thead>
<tr>
<th>Groups*</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases of total cells categorized into each group (a)</td>
<td>18</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>No. of cases giving rise to heat-resistant cells in (a) (b)</td>
<td>3</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Recovery ratio of heat-resistant cells (b/ (%) a)</td>
<td>16.7</td>
<td>38.1</td>
<td>61.1</td>
</tr>
</tbody>
</table>

* Group I includes the organisms in the order of 10^3 or less cells in one ml volume of feces sample.
  Group II includes the organisms in the orders of 10^4 to 10^5 cells.
  Group III includes those in the order of 10^6 or more cells.

Table 2. Number of C. perfringens in human intestines and the age of examinees

<table>
<thead>
<tr>
<th>No. of cells in 1 ml of feces samples</th>
<th>Ca 10^3 or less</th>
<th>Ca 10^4 to Ca 10^5</th>
<th>Ca 10^6 or more</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 to 30</td>
<td>5*</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>41 to 58</td>
<td>1</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>60 to 82</td>
<td>2</td>
<td>4</td>
<td>16</td>
</tr>
</tbody>
</table>

* No. of cases categorized into each level of total cells.

this organism in feces samples and the ages of persons from whom the feces samples were obtained were analyzed, using 54 feces samples. Table 2 indicated that the older the age, the greater was the number of persons carrying heat-resistant strains.

Reinvestigation on the Heat-Resistant Test

As a criterion to differentiate the strains most prevalently inhabiting in human intestines from strains recovered from 100°C for 60 min, we employed a heating at 100°C for 10 min for 48 hr-old cooked meat broth cultures. This was based on the finding that only 5 out of the 133 strains isolated from unheated samples were heat-resistant, when the above-mentioned conditions for cultivation and heating were employed. On the contrary, 105 of the 115 strains recovered from 100°C for 60 min were heat-resistant when examined under the same conditions.

Furthermore, liver broth, brain mush as well as commercial products for anaerobic cultures, such as T.G.C. (Nissui Co. Tokyo) and cooked meat broth (Nissui Co. Tokyo) were examined in this regard with use of 49 strains of C. perfringens recovered from the heat at 100°C for 60 min. These media, however, produced fewer heat-resistant cells than the cooked meat broth prepared in our laboratory. The two commercial products proved to be unemployable because of its particularly poor ability to give rise to heat-resistant cells (Data abbreviated).

Table 3. a-toxigenicity of C. perfringens strains of normal flora in human intestines

<table>
<thead>
<tr>
<th>No. of total strains isolated</th>
<th>a-Toxigenicity (AE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤0.4</td>
</tr>
<tr>
<td>353</td>
<td>169</td>
</tr>
</tbody>
</table>

a-Toxigenicity of C. perfringens Strains as a Normal Flora of Human Intestines

An estimation was performed on the a-toxigenicity of C. perfringens strains most commonly inhabiting in human intestines, utilizing 353 strains isolated from the plates directly smeared by feces samples. All of these strains were obtained from 119 samples during the experiments mentioned above. The result shown in Table 3 indicates that the toxigenicity of 353 strains mainly ranged between 0.05 and 0.9 AE. Also, the a-toxigenicity of 20 C. perfringens strains recovered from feces samples heated at 100°C for 60 min was estimated and proved to be 0.1 AE or less. This confirms the finding of Yamagishi et al. [17] that the high temperatures applied to specimens
gave rise to strains with attenuated or lost \( \alpha \)-toxigenicity.

**DISCUSSION**

Turner [15], demonstrated heat-resistant strains of *C. perfringens* in 63% of feces samples from Chinese in Hong Kong who were not affected with *C. perfringens* food poisoning. Similar high ratios of normal feces samples in Japan have been reported by several investigators [1, 2, 8, 10]. Our present finding indicates that the predominant number of *C. perfringens* cells in normal human intestines to include the organisms resistant to the heat at 100°C for 60 min were sensitive to the heat at 100°C for 10 min. This implies a possibility that food poisoning due to heat-resistant strains might be detectable by examining the heat-resistance of the organisms prevailing in human intestines. Asakawa et al. [3] and Yasukawa et al. [18], when they encountered outbreaks of *C. perfringens* food poisoning, isolated the organisms by directly smearing the sample suspensions on plate agars, while the heat-resistance of these isolates were examined by culturing in cooked meat broth for 48 hr according to our methods [12, 17]. They found that all of these strains were resistant to the heat applied.

Although one of the most significant criteria for the etiology of a food poisoning outbreak would be the discovery of an identical serotype among the strains isolated; Hall et al. [5] demonstrated that over 50% of the *C. perfringens* strains isolated in an outbreak were untypable with Hobbs’ serotype system. Recently Sutton et al. [14] disclosed that each gram of feces contained *C. perfringens* cells in the order of \( 10^5 \) or more in the food poisoning and that equal amounts of normal feces samples of British contained approximately \( 10^3 \) (median counts) cells of the organism. However, of the 111 normal Japanese feces samples, 68 gave rise to more than \( 10^5 \) cells and 14 included more than \( 10^7 \) cells of the organism. It should be noted that Sutton et al. used one gram of feces samples and that we used one ml volume of the samples. No clinical symptom could be found in the latter 14 cases. The high figures in the number of *C. perfringens* in Japanese intestines appear to be closely correlated with the high recovery ratios of the organism resistant to 100°C for 60 min. Since the heat-sensitive *C. perfringens* cells prevailing in normal feces appear to be genetically different from the strains recovered from the heating at 100°C for 60 min, the manner in which the increasing numbers of the total cells were related with the high recovery ratios of the heat-resistant cells, still remains to be studied.

During these experiments, strains of *C. perfringens* isolated from unheated feces samples were examined for their \( \alpha \)-toxigenicities. They mainly ranged between 0.05 and 0.9 AE. This confirms the finding obtained in Britain [13]. In one case, when one of our laboratory staff was contacted a severe diarrhoea, we examined \( \alpha \)-toxigenicity of the 8 strains obtained from a directly smeared plate culture. All of them exhibited such a high \( \alpha \)-toxigenicity of 2 AE or units approximate to it. Further studies were performed in the laboratory of the University clinic and we encountered 5 cases where the strains with higher than 2 AE \( \alpha \)-toxigenicity could be demonstrated. In 3 cases of the 5, gastrointestinal complaints or symptoms were observed. These patients were free from the organisms of pathogenic Enterobacteriaceae. The clinical significance in enterocolitis of the \( \alpha \)-toxigenicity, however, is still open to doubt.
REFERENCES


