Most Probable Number Method Combined with Nested Polymerase Chain Reaction for Detection and Enumeration of Enterotoxigenic Clostridium perfringens in Intestinal Contents of Cattle, Pig and Chicken

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ABSTRACT. The most probable number (MPN) method combined with a nested polymerase chain reaction (nested PCR) for the detection and enumeration of enterotoxigenic Clostridium perfringens in the intestinal contents of cattle, pig and chicken was examined. Ten-fold serial dilutions of samples were added to three tubes of enrichment medium, which were incubated at 37°C for 20–24 hr, and the C. perfringens enterotoxin gene was detected by nested PCR from the enrichment culture without isolating the organism. The results obtained by this method with artificially contaminated intestinal contents were significantly correlated with those obtained by a plate count method. When the method was applied to the detection and enumeration of indigenous enterotoxigenic C. perfringens, the organism was found in two, two and three samples of 10 intestinal contents of cattle, pig and chicken, respectively. Most of the positive samples contained fewer than 10 MPN/g of enterotoxigenic C. perfringens, except one sample of chicken, which contained $1.5 \times 10^7$ MPN/g. The MPN method combined with nested PCR is easy to perform and may be a useful tool for the detection and enumeration of enterotoxigenic C. perfringens in intestinal contents. — KEY WORDS: Clostridium perfringens, enterotoxin, MPN, PCR.


Meat is suspected of being the most likely vehicle causing food poisoning due to Clostridium perfringens [13], which is widely distributed in the intestinal contents of domestic livestock [5, 11, 17, 18]. C. perfringens of livestock intestinal contents origin contaminate carcasses and meat during dressing in slaughterhouses or poultry processing plants [2, 3, 16]. It is well known that the symptoms of food poisoning are caused by an enterotoxin produced by enterotoxigenic C. perfringens [14]. However, only a relatively small fraction of C. perfringens isolated from the intestinal contents of domestic livestock may be enterotoxigenic [11, 15, 18]. In epidemiological investigations, therefore, enumeration of enterotoxigenic C. perfringens, rather than the total numbers of C. perfringens, in the intestinal contents of domestic livestock is important. Several methods have been developed for the enumeration of C. perfringens [1, 4, 6, 7, 10, 12], but, enterotoxigenic C. perfringens cannot be distinguished from nonenterotoxigenic C. perfringens by these methods. Therefore, a simple method which can enumerate enterotoxigenic C. perfringens in intestinal contents containing both enterotoxigenic and nonenterotoxigenic C. perfringens is required. We have reported a method of nested polymerase chain reaction (nested PCR) for detecting low levels of enterotoxigenic C. perfringens in animal feces [9]. This method could detect fewer than 10 colony forming unit per g (CFU/g) of enterotoxigenic C. perfringens in animal feces containing both enterotoxigenic and nonenterotoxigenic C. perfringens without isolation of the organism.

In this study, we examined the most probable number (MPN) method combined with a nested PCR for the detection and enumeration of enterotoxigenic C. perfringens. This method was compared with a plate count method by using artificially contaminated intestinal contents, and was also applied to the detection and enumeration of indigenous enterotoxigenic C. perfringens in the intestinal contents of cattle, pigs and chickens. The advantages of the MPN method combined with nested PCR for the detection and enumeration of enterotoxigenic C. perfringens are also discussed. To our knowledge, this is the first report about the quantitative study of enterotoxigenic C. perfringens in intestinal contents of domestic livestock.

MATERIALS AND METHODS

Strains: Enterotoxigenic C. perfringens (NCTC 8238 and NCTC 8239) were provided by Dr. T. Itoh, Tokyo Metropolitan Research Laboratory of Public Health. Nonenterotoxigenic C. perfringens (N101) was isolated from cattle feces at Shizuoka Prefectural Western Meat Inspection Center [9].

Detection and enumeration of enterotoxigenic C. perfringens in artificially contaminated intestinal contents: The intestinal contents of cattle, pigs and chickens were collected at a slaughterhouse and a poultry processing plant. After confirming the absence of C. perfringens, a 20-g portion of each intestinal content was suspended in 180 ml of liquid thioglycolate medium II (without agar: Nissui Pharmaceutical Co., Ltd., Tokyo), and homogenized for 1 min with a Stomacher 400 (Organco Co., Ltd., Tokyo). An enterotoxigenic C. perfringens cell suspension was prepared and the CFU of the cell suspension were determined by the plate count method. Portions of 19 ml of the sample homogenates were transferred to sterile tubes and inoculated...
with 1 ml of an appropriate dilution of enterotoxigenic C. perfringens cell suspension to obtain from 10^0 to 10^6 CFU/ml of homogenates. In some cases, both enterotoxigenic C. perfringens and 10^3 CFU/ml levels of nonenterotoxigenic C. perfringens were added to the same sample homogenates.

The number of enterotoxigenic C. perfringens of the artificially contaminated sample homogenate was enumerated as follows. A ten-fold serial dilution of each sample homogenate was carried out, and 1 ml of each homogenate and the serial dilutions were transferred to three tubes of liquid thioglycolate medium II (10 ml), which were then incubated at 37°C for 20–24 hr.

(i) MPN method combined with nested PCR (Nested-PCR-MPN). Each incubated culture (1 ml) mentioned above was centrifuged at 3,000 × g for 1 min, and the sediment was suspended with 100 µl of sterile distilled water. The suspension was boiled for 5 min and centrifuged at 3,000 × g for 1 min. The supernatant was applied to the nested PCR assay described previously [9]. The number of tubes which showed positive results for the nested PCR were counted as containing enterotoxigenic C. perfringens. Then, the number of enterotoxigenic C. perfringens was determined as MPN per ml of homogenate.

(ii) MPN method by isolating enterotoxigenic C. perfringens (Conventional-MPN). Portions of 0.1 ml of each incubated culture mentioned above were spread on CW agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo) with egg-yolk, which were then incubated anaerobically at 37°C for 20–24 hr. Three to five suspected colonies grown on a CW egg-yolk agar plate were confirmed for C. perfringens, and the enterotoxigenicity of the C. perfringens isolates was determined by a single PCR [9]. The number of tubes from which enterotoxigenic C. perfringens was isolated was counted, and the number of enterotoxigenic C. perfringens was determined as MPN per ml of homogenate.

Detection and enumeration of indigenous enterotoxigenic C. perfringens in intestinal contents: Sample homogenates and serial dilutions of the intestinal contents of cattle, pig and chicken were prepared as described above, except that the sample homogenates were not inoculated with C. perfringens. The sample homogenate (10 ml and 1 ml) and serial dilutions (1 ml) were transferred to three tubes of liquid thioglycolate medium II, which were then incubated at 37°C for 20–24 hr. Then, the numbers of enterotoxigenic C. perfringens were enumerated by Nested-PCR-MPN and Conventional-MPN.

RESULTS

Detection and enumeration of enterotoxigenic C. perfringens in artificially contaminated intestinal contents: The numbers of enterotoxigenic C. perfringens enumerated by Nested-PCR-MPN and Conventional-MPN in artificially contaminated sample homogenates and those of CFU obtained by using pure culture of C. perfringens were plotted together on log-log paper.

When the sample homogenates were inoculated with only enterotoxigenic C. perfringens, regression analysis indicated that there was a significant correlation between the numbers of enterotoxigenic C. perfringens enumerated by Nested-PCR-MPN and those of CFU (Fig. 1, r value=0.90). Furthermore, in the sample homogenates inoculated with both enterotoxigenic and nonenterotoxigenic C. perfringens, the numbers of enterotoxigenic C. perfringens enumerated by Nested-PCR-MPN were also significantly correlated with those of CFU (Fig. 2, r value=0.89). By Conventional-MPN, the numbers of enterotoxigenic C. perfringens were significantly correlated with those of CFU in the sample homogenates inoculated with only enterotoxigenic C. perfringens (data not shown), however, small numbers of enterotoxigenic C. perfringens were not detected in the sample homogenates inoculated with both enterotoxigenic and nonenterotoxigenic C. perfringens (Fig. 3).

There were no appreciable differences in the discriminative ability of Nested-PCR-MPN between strains NCTC 8238 and NCTC 8239 or between cattle, pig and chicken animal species from which intestinal contents were obtained.

Detection and enumeration of indigenous enterotoxigenic C. perfringens in intestinal contents: The intestinal contents of cattle, pigs and chickens were examined by Nested-PCR-MPN or Conventional-MPN for the detection and enumeration of indigenous enterotoxigenic C. perfringens

![Fig. 1. Correlation between the numbers of enterotoxigenic C. perfringens enumerated by the MPN method combined with nested PCR (Nested-PCR-MPN) and those of CFU in sample homogenates inoculated with 10^0–10^6 CFU/ml levels of enterotoxigenic C. perfringens. Symbols: □ and □, intestinal contents of cattle inoculated with enterotoxigenic C. perfringens NCTC 8238 and NCTC 8239, respectively; □ and □, intestinal contents of pig inoculated with enterotoxigenic C. perfringens NCTC 8238 and NCTC 8239, respectively; □ and □, intestinal contents of chicken inoculated with enterotoxigenic C. perfringens NCTC 8238 and NCTC 8239, respectively.](image-url)
The numbers of enterotoxigenic *C. perfringens* were <10 MPN/g, <10 MPN/g and <10–1.5 × 10^2 MPN/g of intestinal contents of cattle, pig and chicken, respectively. The numbers of enterotoxigenic *C. perfringens* were <10 MPN/ml, <10 MPN/ml and <10–1.5 × 10^2 MPN/ml of enterotoxigenic *C. perfringens* and with 10^3 CFU/ml levels of nonenterotoxigenic *C. perfringens*. Symbols are same as Fig. 1 and each sample was inoculated with nonenterotoxigenic *C. perfringens* N101 in addition to the enterotoxigenic strain.

**DISCUSSION**

We have reported that nested PCR combined with enrichment culture of the sample is a useful tool for detecting low levels of enterotoxigenic *C. perfringens* in samples containing a mixed population of enterotoxigenic and nonenterotoxigenic *C. perfringens* [9]. In this study, the sensitivity and specificity of Nested-PCR-MPN for the detection and enumeration of enterotoxigenic *C. perfringens* in artificially contaminated intestinal contents were ascertained by comparison with the plate count method. By Nested-PCR-MPN, the number of enterotoxigenic *C. perfringens* could be enumerated without isolating the organism in intestinal contents inoculated with enterotoxigenic *C. perfringens*, with or without a nonenterotoxigenic strain, and the numbers of enterotoxigenic *C. perfringens* correlated significantly with those of CFU obtained by the plate count method (Figs. 1 and 2).

**Table 1.** Detection and enumeration of indigenous enterotoxigenic *C. perfringens* in intestinal contents of cattle, pig and chicken

<table>
<thead>
<tr>
<th>Source</th>
<th>Number of samples examined</th>
<th>Number of samples positive</th>
<th>Number of enterotoxigenic <em>C. perfringens</em> cells (MPN/g)</th>
<th>Number of samples positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;10 MPN/g</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>10–10^2 MPN/g</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;10^3 MPN/g</td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Pig</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Chicken</td>
<td>10</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
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(Fig. 2). Correlation between the numbers of enterotoxigenic *C. perfringens* enumerated by Conventional-MPN and those of CFU in sample homogenates inoculated with 10^0–10^6 CFU/ml levels of enterotoxigenic *C. perfringens* and with 10^3 CFU/ml levels of nonenterotoxigenic *C. perfringens*. Symbols are same as Fig. 2.

(Fig. 3). Correlation between the numbers of enterotoxigenic *C. perfringens* enumerated by Conventional-MPN and those of CFU in sample homogenates inoculated with 10^0–10^6 CFU/ml levels of enterotoxigenic *C. perfringens* and with 10^3 CFU/ml levels of nonenterotoxigenic *C. perfringens*. Symbols are same as Fig. 2.
Furthermore, a small number of \textit{C. perfringens} cannot be detected by the plate count method because only a small amount of sample can be applied to the plate [4, 10]. The MPN method has been used to enumerate \textit{C. perfringens} [1, 2, 4, 8], however, these methods were not applied to the enumeration of enterotoxigenic \textit{C. perfringens} but total \textit{C. perfringens} (enterotoxigenic and nonenterotoxigenic) because each of the organisms concerned with the enterotoxigenicity could not be distinguished by these MPN methods. For enumeration of enterotoxigenic \textit{C. perfringens}, Conventional-MPN requires the isolation of the organism from the sample and testing for enterotoxigenicity of the isolates, however, our results indicate that the sensitivity of Conventional-MPN for the detection and enumeration of enterotoxigenic \textit{C. perfringens} is decreased by the presence of nonenterotoxigenic \textit{C. perfringens} (Fig. 3). Furthermore, when Nested-PCR-MPN and Conventional-MPN were applied to the detection and enumeration of indigenous enterotoxigenic \textit{C. perfringens} in the intestinal contents of cattle, pig and chicken, the organism was found in 20–30% of samples (the number: $\times 10^2 \text{ MPN/g}$) by Nested-PCR-MPN, but was not found by Conventional-MPN (Table 1). These results indicate the advantage of Nested-PCR-MPN.

The Nested-PCR-MPN described here is sensitive and specific, and the sensitivity and specificity were not affected by the presence of nonenterotoxigenic \textit{C. perfringens}. Furthermore, this method does not require laborious procedures such as anaerobic incubation, isolating the organism, and testing the enterotoxigenicity of the many isolates. Thus, Nested-PCR-MPN may be a useful tool for the detection and enumeration of enterotoxigenic \textit{C. perfringens} in intestinal contents containing both enterotoxigenic and nonenterotoxigenic \textit{C. perfringens}.

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**REFERENCES**