Most Probable Numbers of Enterotoxigenic \textit{Clostridium perfringens} in Intestinal Contents of Domestic Livestock Detected by Nested PCR

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**ABSTRACT.** The incidence and numbers of enterotoxigenic \textit{Clostridium perfringens} in the intestinal contents of cattle, swine and broiler chickens were determined and compared with those of total (enterotoxigenic and nonenterotoxigenic) \textit{C. perfringens}. The method used for the enumeration of enterotoxigenic \textit{C. perfringens} consisted of a combination of the most probable number (MPN) method and a nested polymerase chain reaction after enrichment culture of the sample. Enterotoxigenic \textit{C. perfringens} was found in 26% (4.0 × 10^4–4.3 × 10^5 MPN/100 g), 22% (4.0 × 10^2–2.3 × 10^4 MPN/100 g) and 40% (4.0 × 10^2–2.4 × 10^5 MPN/100 g) of intestinal contents of 50 head each of cattle, swine and broiler chickens, respectively. Whereas, total \textit{C. perfringens} was found in 76% (9.0 × 10^2–7.5 × 10^6 MPN/100 g), 44% (7.0 × 10–4.3 × 10^4 MPN/100 g) and 80% (4.3 × 10^2–9.3 × 10^5 MPN/100 g) of intestinal contents of 50 head each of cattle, swine and broiler chickens, respectively, by the conventional MPN method. In all cases, enterotoxigenic cells were not dominant in the population of \textit{C. perfringens}: a small number of enterotoxigenic cells of \textit{C. perfringens} co-existed with a large number of nonenterotoxigenic cells in the same sample. The ratios of enterotoxigenic \textit{C. perfringens} cells to total \textit{C. perfringens} cells were 1/10–1/10^7. — KEY WORDS: \textit{Clostridium perfringens}, enterotoxin, livestock, MPN, PCR.

\textit{Clostridium perfringens} is an important causative agent of human food poisoning. Meat, which is often contaminated with \textit{C. perfringens}, is suspected as a major source of food poisoning due to \textit{C. perfringens} [8]. Among domestic livestock, high fecal carriage of \textit{C. perfringens} has been reported [3, 7, 12, 13], and \textit{C. perfringens} of intestinal origin may contaminate carcasses and meat during processing in the slaughterhouse [1, 2, 11]. The symptoms associated with \textit{C. perfringens} food poisoning are caused by an enterotoxin produced by enterotoxigenic \textit{C. perfringens} [9]. Therefore, enumeration of enterotoxigenic \textit{C. perfringens} in the intestinal contents of domestic livestock, rather than that of total numbers of \textit{C. perfringens}, needs to be considered in relation to food poisoning. There are several reports of the incidence of enterotoxigenic \textit{C. perfringens} in fecal samples of domestic livestock [3, 4, 7, 10, 12–14]. To our knowledge, however, there is little information about the quantitative study of enterotoxigenic \textit{C. perfringens} in fecal samples of domestic livestock. We have reported a method of nested polymerase chain reaction (nested PCR) for detecting low levels of enterotoxigenic \textit{C. perfringens} in animal feces containing both enterotoxigenic and nonenterotoxigenic \textit{C. perfringens} without isolating the organism [5]. Furthermore, the most probable number (MPN) method combined with the nested PCR method has been applied to the detection and enumeration of enterotoxigenic \textit{C. perfringens} in the intestinal contents of cattle, swine and broiler chickens [6]. In these reports, however, only a small number of samples were tested, and the numbers of nonenterotoxigenic \textit{C. perfringens} were not examined. In this study, the incidence and the number of enterotoxigenic \textit{C. perfringens} in the intestinal contents of cattle, swine and broiler chickens were determined and compared with those of total (enterotoxigenic and nonenterotoxigenic) \textit{C. perfringens}.

**MATERIALS AND METHODS**

\textit{Samples:} Intestinal contents were obtained from 50 head each of slaughtered cattle, swine and broiler chickens. Three to five intestinal contents were obtained from animals which were fed on the same farm. All animals were healthy and showed no signs of enteritis or other illness. Each sample was placed in a sterile plastic bag, transported to the laboratory in a chilled box and analyzed on the day of slaughter.

\textit{Enumeration of enterotoxigenic \textit{C. perfringens}:} A 10-g portion of each sample was aseptically placed in a filter stomacher bag and homogenized for 1 min in 90 ml of liquid thioglycolate medium II (without agar: Nissui Pharmaceutical Co., Ltd., Tokyo) with a stomacher. Then 10-fold serial dilutions of each sample homogenate was carried out. Three 10 ml portions of homogenate were each transferred to 40 ml of liquid thioglycolate medium II, and three 1 ml portions of homogenate and each of the serial dilutions were each transferred to 10 ml of liquid thioglycolate medium II. The culture tubes were then incubated at 37°C for 20–24 hr. One ml of each incubated
culture was centrifuged at 3,000 x 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 x g for 1 min. The supernatant was applied to the nested PCR assay described previously [5]. The number of tubes showing a positive result for the nested PCR was counted as containing enterotoxigenic C. perfringens. Then, the number of enterotoxigenic C. perfringens was determined from the MPN table [6].

**Enumeration of total C. perfringens:** One hundred µl of each incubated culture of liquid thioglycolate medium II was spread on a CW agar plate (Nissui Pharmaceutical Co., Ltd., Tokyo) with egg-yolk, which was 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 to be C. perfringens. The number of tubes in which C. perfringens was detected was counted, and the number of total C. perfringens was determined from the MPN table.

**RESULTS**

**Incidence and number of enterotoxigenic C. perfringens:** The frequency distribution of enterotoxigenic C. perfringens present in the intestinal contents of domestic livestock is shown in Table 1. The microorganism was found in 13 (26.0%), 11 (22.0%) and 20 (40.0%) intestinal contents of cattle, swine and broiler chickens, respectively. Of the 13 positive samples from cattle, 10 samples contained fewer than 10^2 MPN/100 g, two samples contained 2.3 x 10^2 MPN/100 g and one sample contained 4.3 x 10^2 MPN/100 g. Of the 11 positive samples from swine, 10 samples contained fewer than 10^2 MPN/100 g and one sample contained 2.3 x 10^3 MPN/100 g. More than half of the 20 positive samples (12 samples) from broiler chickens were found to contain more than 10^2 MPN/100 g (six samples 10^2 to 10^3 MPN/100 g, five samples 10^3 to 10^4 MPN/100 g and one sample 2.4 x 10^4 MPN/100 g).

**Incidence and number of total C. perfringens:** The frequency distribution of total C. perfringens is shown in Table 2. The microorganism was found in 38 (76.0%), 22 (44.0%) and 40 (80.0%) intestinal contents of cattle, swine and broiler chickens, respectively. The numbers of total C. perfringens were distributed from 9.0 x 10^2 to 7.5 x 10^6 MPN/100 g, 7.0 x 10^2 to 4.3 x 10^6 MPN/100 g and 4.3 x 10^2 to 9.3 x 10^7 MPN/100 g in the intestinal contents of cattle, swine and broiler chickens, respectively.

**Ratios of enterotoxigenic cells to total C. perfringens cells:** The ratios of enterotoxigenic cells to total C. perfringens cells are shown in Table 3.
Enterotoxigenic *C. perfringens* cells present in the samples in which the enterotoxin gene was detected are shown in Table 3. The ratios of enterotoxigenic cells to total *C. perfringens* cells were 1/10–1/10^5. In all cases, the enterotoxigenic cells were not dominant in the population of *C. perfringens*: a small number of enterotoxigenic cells of *C. perfringens* co-existed with a large number of nonenterotoxigenic cells in the same samples.

**DISCUSSION**

In this study, enterotoxigenic *C. perfringens* was found in 22 to 40% of 50 each of cattle, swine and broiler chickens intestinal contents by the MPN method combined with the nested PCR after enrichment culture of the sample (Table 1). With such a high incidence of enterotoxigenic *C. perfringens* in the intestinal contents of slaughtered animals, carcasses may readily be contaminated with this microorganism during processing at the slaughterhouse or the poultry processing plant.

The incidence of enterotoxigenic *C. perfringens* in fecal samples of cattle, swine and chickens have been reported as ranging from 0% to 22% in other investigations [3, 4, 7, 10, 12–14]. The discrepancies between our results and others may be due to the differences in the methods used. In our investigation, the MPN method combined with the nested PCR was very sensitive and specific, and a small number of enterotoxigenic *C. perfringens* could be found in samples with a large number of nonenterotoxigenic cells [6]. In other investigations, *C. perfringens* was isolated from samples and then the enterotoxigenicity of the isolates was tested for the detection of enterotoxigenic *C. perfringens*. However, our results indicate that a small number of enterotoxigenic cells co-exist with a large number of nonenterotoxigenic cells. Therefore, if a limited number of isolates from fecal samples are picked for screening enterotoxigenicity, enterotoxigenic strains may not be detected.

Most of the enterotoxigenic *C. perfringens* positive samples from cattle and swine contained fewer than 10^2 enterotoxigenic cells per 100 g of intestinal contents. Whereas, more than half of the enterotoxigenic *C. perfringens* positive samples from broiler chickens contained in excess of 10^5 enterotoxigenic cells per 100 g of intestinal contents, which was highest incidence found. Two explanations may be offered for this finding. First, the relatively high incidence and levels of enterotoxigenic *C. perfringens* in intestinal contents of chickens may reflect the environmental conditions in which broiler chickens are fed. Broiler chickens are usually kept in large numbers in poultry houses with low ventilation. Consequently, they are subjected to frequent exposure to excrement, and cross infection may have happened during feeding. Another explanation is that broiler chickens may be more susceptible than cattle and swine to intestinal colonization by enterotoxigenic *C. perfringens* regardless of the degree of exposure to these bacteria. However, a limited number of samples were examined in this study, and further work on the incidence and level of enterotoxigenic *C. perfringens* in the intestinal contents of chickens should be done before definite conclusions are drawn.

As a result of this experiment, it could be concluded that a small number of enterotoxigenic *C. perfringens* co-exist with a large number of nonenterotoxigenic *C. perfringens* in the intestinal contents of livestock, and the number of enterotoxigenic cells can be enumerated by the MPN method combined with the nested PCR regardless of the small population ratio of enterotoxigenic cells to nonenterotoxigenic cells. To our knowledge, this is the first report about the ratios of enterotoxigenic and nonenterotoxigenic cells of *C. perfringens* in intestinal contents of livestock.

**REFERENCES**

