Epidemiol. Infect. (2008), **137**, 108–113. © 2008 Cambridge University Press
doi:10.1017/S0950268808000794 Printed in the United Kingdom

Spread of a large plasmid carrying the *cpe* gene and the *tcp* locus amongst *Clostridium perfringens* isolates from nosocomial outbreaks and sporadic cases of gastroenteritis in a geriatric hospital

S. KOBAYASHI[1,4], A. WADA[1]*, S. SHIBASAKI[2], M. ANNAKA[2], H. HIGUCHI[2], K. ADACHI[2], N. MORI[3], T. ISHIKAWA[3], Y. MASUDA[3], H. WATANABE[1], N. YAMAMOTO[4,5], S. YAMAOKA[4] AND T. INAMATSU[3]

[1] *Department of Bacteriology, National Institute of Infectious Diseases, Tokyo, Japan*
[2] *Clinical and Research Laboratory and* [3] *Department of Infectious Diseases, Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan*
[4] *Department of Virology, Tokyo Medical and Dental University, Tokyo, Japan*
[5] *AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan*

(*Accepted 11 April 2008; first published online 19 May 2008*)

SUMMARY

To investigate two clusters of diarrhoea cases observed in our geriatric hospital wards, the faecal specimens were analysed. Reversed passive latex agglutination assay revealed that 63·2% and 41·7% of the faecal specimens from each cluster were positive for *Clostridium perfringens* enterotoxin. PCR assay revealed that 71·4% and 68·8% of *C. perfringens* isolates from each cluster were positive for the enterotoxin gene (*cpe*). These observations suggested that both the clusters were outbreaks caused by enterotoxigenic *C. perfringens*. Subsequent pulsed-field gel electrophoresis analysis revealed that the two outbreaks were caused by different *C. perfringens* isolates. However, these outbreak isolates as well as other sporadic diarrhoea isolates shared a 75-kb plasmid on which the *cpe* gene and the *tcp* locus were located. The 75-kb plasmid had horizontally spread to various *C. perfringens* isolates and had caused outbreaks and sporadic infections. However, the site and time of the plasmid transfer are unclear.

INTRODUCTION

Clostridium perfringens is a Gram-positive, anaerobic spore-forming bacterium, and *C. perfringens* type A is a causative agent of food poisoning and non-foodborne human gastrointestinal diseases [1]. *C. perfringens* is commonly found in the gastrointestinal tract of mammals, as well as in soil and sewage. The diarrhoea and cramps, that comprise the typical clinical symptoms of this human gastrointestinal disease, are induced by a single 35-kDa polypeptide known as *C. perfringens* enterotoxin (CPE) [2–4]. The knock-out mutant of *cpe*, i.e. the gene encoding CPE, could not induce rabbit ileal loop fluid accumulation and intestinal histopathological damage [5]. Its synthesis is under the strict positive control of sporulation in the sporulation medium [5–7] and thus in the intestinal environment. Brett *et al.* reported that CPE-producing *C. perfringens* is responsible for 6·8% of all cases of sporadic diarrhoea [8]. There are multiple reports of non-foodborne human gastrointestinal diseases in elderly people [9–14]. It is reported that the intestinal flora of elderly people includes a higher number of *C. perfringens* than that of younger people [15].

In this study, we analysed *C. perfringens* isolates which were derived from clusters of gastroenteritis cases in our geriatric hospital. We found that the

* Author for correspondence: Dr A. Wada, Department of Bacteriology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan.
(Email: awada@nih.go.jp)

clusters were outbreaks of enterotoxigenic *C. perfringens* infection. In the outbreak and sporadic isolates, *cpe* was plasmid borne. The experimental data showed that the same *cpe*-carrying plasmid had spread amongst the *C. perfringens* isolates.

MATERIALS AND METHODS

Clinical specimens and culture conditions

All faecal specimens from diarrhoea cases were examined routinely by Gram staining and by culture for the detection of *Salmonella*, *Shigella*, *Campylobacter*, *Vibrio*, *Aeromonas* and *Yersinia*. Faecal specimens in which round-ended Gram-positive rods containing spores were observed were analysed for CPE by using a reversed passive latex agglutination assay (RPLA) (Denka Seiken, Tokyo, Japan). The faecal specimens were mixed with 10× volume of saline and centrifuged at 3000 rpm for 20 min. Then the supernatant was serially diluted with diluent in the kit before being mixed with the latex beads. The culture method used for *C. perfringens* detection was as follows. The specimens were mixed with an equal volume of 99·5% alcohol and incubated for 30 min at room temperature. They were then plated on Gifu Anaerobic Medium agar plate (Nissui, Tokyo, Japan) containing 5% egg yolk and cultured at 35 °C for 18–24 h in an anaerobic pouch (AnaeroPack; Mitsubishi Gas Chemical Co., Tokyo, Japan). A lecithinase-positive colony was selected from each plate and tested for haemolytic activity, motility and gas production. The resultant *C. perfringens* isolates were subjected to subsequent analyses.

Polymerase chain reaction (PCR)

The typing of *C. perfringens* isolates was performed by the established multiplex PCR [16]. The *cpe* gene was detected by PCR with primers 3F and 4R according to the procedure of Miyamoto *et al.* [17]. For distinguishing between the chromosomal and plasmid-borne *cpe*, *cpe*-IS sequences were amplified with primers cpe4F, IS1470R1.3, IS1470-likeR1.6 and IS1151R0.8 according Miyamoto *et al.* [17]. A sequence in the *tra* locus, which corresponds to a part of the *orf16* of the putative Tn*916*, was also amplified by primers 69219 (5′-CTTCATAGGATTGCTTCGCTC-3′) and 70672R (5′-CTTATAAATCCACATACAGACCAATACAG-3′) [18]. The *tra* locus and *orf16* have been renamed *tcp* locus and *tcpF* respectively [19], and the new names will be used hereafter. The PCR was performed with ExTaq (Takara Bio, Otsu, Japan). The annealing temperatures were 55 °C, 50 °C and 45 °C for the *cpe*, *cpe*-IS and *tcpF* amplifications, respectively.

Pulsed-field gel electrophoresis (PFGE)

Samples for PFGE were prepared as previously described [13]. In this analysis, 100 μM of thiourea (Sigma, MO, USA) was added to 0·5× Tris-borate-EDTA buffer in order to prevent the degradation of clostridial DNA [20]. The PFGE conditions are described in the figure legends. Southern hybridization analysis was performed after PFGE or conventional gel electrophoresis. The probes were labelled by digoxigenin (Roche, Basel, Switzerland) and used for hybridization according to the manufacturer's instructions.

RESULTS

Analysis of faecal specimens and *C. perfringens* isolates

Our hospital has 646 beds for the primary care of elderly patients. One ward has a total of 40–43 beds: six rooms with six beds, zero or one room with four beds, and three or four rooms with one bed. Medical staff members belong to each ward in principle, but doctors have the opportunity to examine patients in other wards as consultants. Patients also have the chance of having contact with patients from other wards at the central examination unit. The average period of hospitalization is about 18 days. Usually, 0–5 isolations of *C. perfringens* per month are recorded. From the middle of February to the beginning of April 2006, a cluster of diarrhoea cases was observed in ward W10. During this period, 19 faecal specimens from 16 patients in this ward were examined, and 16 *C. perfringens* isolates were obtained from 14 patients. However, the number of *C. perfringens* isolations reverted to the normal level by the middle of April 2006. From the end of July to the beginning of September 2006, another cluster of diarrhoea cases was observed in ward W08. During this period, 25 faecal specimens from 18 patients in this ward were examined, and 16 *C. perfringens* isolates were obtained from 11 patients. Using the RPLA assay for detection of CPE, positive results were obtained in 63·2% (12/19) and 41·7% (10/24)

https://doi.org/10.1017/S0950268808000794 Published online by Cambridge University Press

of faecal specimens from wards W10 and W08, respectively. The positive results were obtained with 1280× or 2560× diluted supernatants of the faecal specimens. Using the PCR assay for detection of the *cpe* gene in the *C. perfringens* isolates, positive results were obtained in 71·4% (10/14) and 68·8% (11/16) of ward W10 and ward W08 isolates, respectively. These PCR results coincided with those of the Southern hybridization analysis by using a *cpe*-PCR product as a probe (see below). The numbers of patients whose faecal samples were positive for CPE and/or contained the *cpe*-positive *C. perfringens* isolates were 12 and nine in wards W10 and W08, respectively. No enteropathogenic bacteria other than *C. perfringens* were isolated in these patients. None of these patients had received antibiotic therapy before the onset of the diarrhoea. Because of the observed *cpe*-PCR-positive rates in our isolates, which were markedly higher than those in normal human *C. perfringens* isolates (see below), we consider the diarrhoea clusters to be outbreaks caused by enterotoxigenic *C. perfringens*.

PFGE and Southern hybridization analysis

In order to clarify the relationship between the two outbreaks, we analysed the 18 ward W10 isolates (14 isolates from the February–April outbreak plus four additional isolates during the July–September period), the 16 ward W08 isolates from the July–September outbreak, and five sporadic isolates from patients in other wards or from an outpatient. All the isolates were type A by multiplex PCR assay for typing [16]. By PFGE analysis with *Sma*I digestion, the restriction profiles of the 28 *cpe*-positive isolates (14 ward W10 isolates, 11 ward W08 isolates and three sporadic isolates) could be classified into seven patterns, when minor variations were considered (Fig. 1*a*, lanes 1, 2, 4, 5, 7, 8, 9). All of the 11 *cpe*-positive ward W08 isolates exhibited identical PFGE patterns (Fig. 1*a*, lane 1). Of the 14 *cpe*-positive ward W10 isolates, nine isolates obtained in the February–April outbreak exhibited identical PFGE patterns (Fig. 1*a*, lane 2), whereas the four isolates obtained during the July–September period exhibited different PFGE patterns (Fig. 1*a*, lanes 5–7) from that of the February–April outbreak isolates (Fig. 1*a*, lane 2). The remaining one isolate, i.e. isolate 210, was obtained in the February–April outbreak period from ward W10, but it possessed a different PFGE pattern (Fig. 1*a*, lane 4) from that of the outbreak isolates from ward W10 (Fig. 1*a*, lane 2). The patterns of the outbreak isolates of wards W08 and W10 were very similar but distinguishable; the migration rates of the second and fourth bands slightly differed from each other (shown by white arrowheads in Fig. 1*a*, lane 1). The PFGE patterns of two sporadic *cpe*-positive isolates from other wards were different from the isolates of wards W10 and W08 (Fig. 1*a*, lanes 8, 9). The remaining one sporadic isolate revealed a pattern that was indistinguishable from that of the ward W10 outbreak isolate (data not shown). This isolate was from an outpatient who had been hospitalized in ward W10 (her hospitalization period overlapped with the outbreak period of ward W10).

Subsequent Southern hybridization analysis using a *cpe*-PCR product from isolate 101 as a probe showed that none of these *Sma*I fragments reacted with the probe. Instead, the origins of the *cpe*-positive isolates yielded positive signals (data not shown). When *Not*I was used for digesting the *C. perfringens* DNAs, 75-kb fragments were obtained in all but one *cpe*-positive isolate (Fig. 1*b*). The *cpe* probe in turn reacted with these 75-kb fragments (Fig. 1*c*). The one exceptional isolate, i.e. isolate 218, had a slightly larger *Not*I fragment (Fig. 1*b*, lane 7), which was also positive for the *cpe* hybridization signal (Fig. 1*c*, lane 7). The *Not*I fragment of isolate 202 was slightly smaller than 75 kb (Fig. 1*b*, lane 3), but it was negative for the *cpe*-hybridization signal (Fig. 1*c*, lane 3). Sporadic isolate 305, whose *Sma*I–PFGE pattern (Fig. 1*a*, lane 10) was indistinguishable from that of the outbreak isolates from ward W08 (Fig. 1*a*, lane 1), did not have a large *Not*I fragment (Fig. 1*b*, lane 10) and was also negative for the *cpe*-hybridization signal (Fig. 1*c*, lane 10). Not by *Sma*I digestion, but by *Not*I digestion, the DNA molecules on which the *cpe* gene was located were linearized and came to migrate into the gel. This observation denoted that the *cpe* gene is not located on the chromosome but on the 75-kb plasmid in our isolates.

PCR analysis of IS sequence

The *cpe* gene on the chromosome is typically associated with IS1470, whereas this gene on plasmids has been reported as located in two forms: one associated with IS1470-like and the other with IS1151 [21, 22]. Miyamoto *et al.* have developed a PCR test for the identification of the IS1470-associated chromosomal *cpe*, the IS1151-associated episomal *cpe* and the

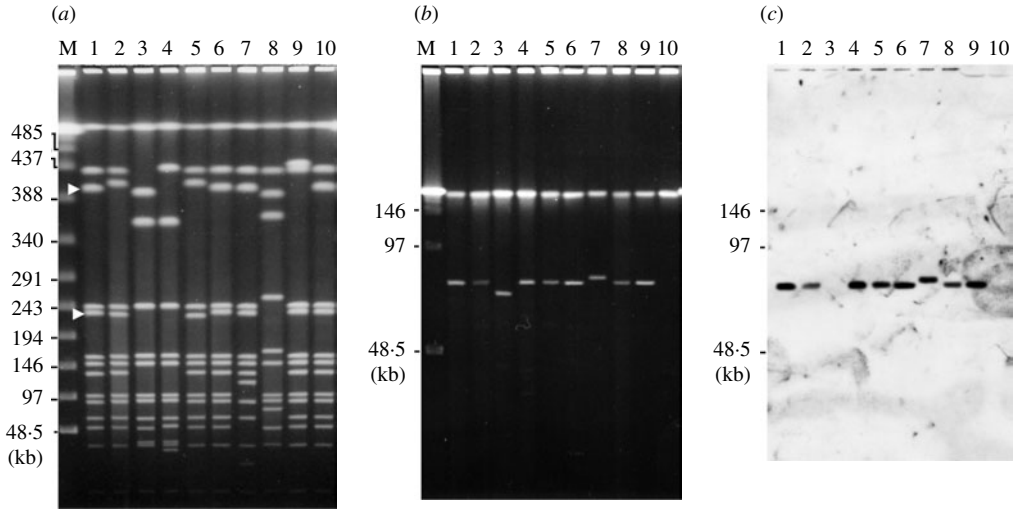

Fig. 1. (*a*) Comparison of *Sma*I-digested chromosomal fragments of the representative *C. perfringens* isolates. The names of isolates are as follows: lane 1, 101; lane 2, 201; lane 3, 202; lane 4, 210; lane 5, 215; lane 6, 216; lane 7, 218; lane 8, 302; lane 9, 304; lane 10, 305. Isolate 101 is from ward W08; isolates 201, 202, 210, 215, 216 and 218 are from ward W10; and isolates 302, 304 and 305 are from other wards. All isolates except isolates 202 and 305 were *cpe* positive by PCR assay. Two fragments of isolate 101 whose migration rates differ from those of the corresponding fragments of isolate 201 are shown by white arrowheads. 'M' indicates a size maker of lambda concatemers. The sizes of the marker are shown on the left side of the image. The strength of the electric field was 6 V/cm. The switching interval of the electric field was ramped from 20 s to 25 s for 18 h. (*b*) Comparison of *Not*I-digested DNA fragments of *C. perfringens* isolates. The isolate in each lane is the same as panel (*a*). The strength of the electric field was the same as panel (*a*). The switching interval of the electric field was ramped from 0·5 s to 5 s for 20 h. (*c*) Southern hybridization analysis of the *Not*I-digested DNA fragments shown in panel (*b*). The *cpe* gene of isolate 101 was amplified with primers 3F and 4R, and the resulting PCR product was used as a probe.

IS1470-like-associated episomal *cpe* [17]. In this PCR test, the *cpe* gene was shown to be associated with IS1151 in all our outbreak and sporadic isolates (data not shown). According to the published nucleotide sequence of a large plasmid harbouring *cpe*-IS1151, this plasmid has a single *Not*I site [18], which is comparable with our PFGE analysis result.

Comparison of the 75-kb plasmids

To clarify whether or not the 75-kb plasmids in the various *C. perfringens* isolates were identical, we performed conventional gel electrophoresis with *Pvu*II digestion of the genomic DNAs, and subsequent Southern hybridization analysis by using the 75-kb *Not*I fragment of isolate 101 as a probe. The probe hybridized to the same four bands of the *cpe*-positive isolates (Fig. 2). The *Pvu*II restriction profile of isolate 218, which had a slightly larger plasmid than the others (Figs 1*b*, *c*, lane 7), was indistinguishable from that of the 75-kb plasmid (Fig. 2, lane 7). However, isolate 202 (Fig. 2, lane 3), which has a *cpe*-negative plasmid (see above), showed a different hybridization pattern from the others.

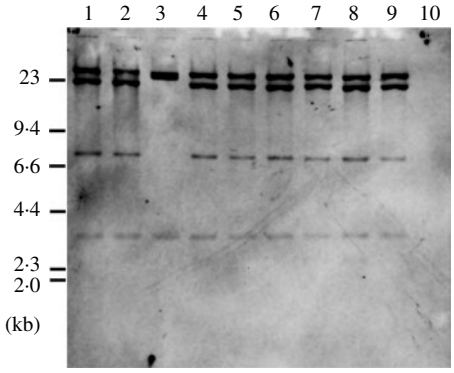

Fig. 2. Southern hybridization analysis of *Pvu*II-digested genomic DNAs of *C. perfringens* isolates. The DNAs were separated in 0·7% agarose gel by conventional gel electrophoresis and hybridized with the digoxigenin-labelled 75-kb *Not*I fragment of isolate 101. The isolate in each lane is the same as that of Figure 1*a*. The sizes of the marker are shown on the left side of the hybridization panel.

The *tcpF* gene PCR

We used two primers, namely, 69219 and 70672R, for assessing the presence of the *tcpF* gene in our isolates. This gene has been shown to be essential for the conjugative transfer of pCW3 [19]. All of the

https://doi.org/10.1017/S0950268808000794 Published online by Cambridge University Press

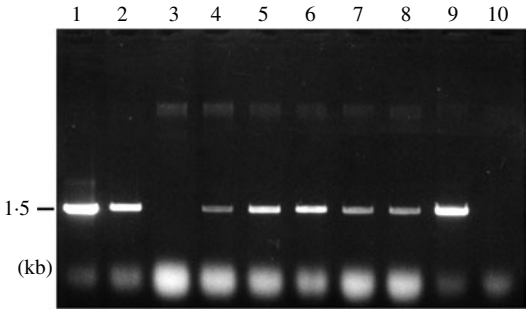

Fig. 3. Amplification of *tcpF*-specific DNA fragments. The isolate in each lane is the same as that of Figure 1*a*. The size of the PCR product is shown on the left side of the image.

cpe-positive isolates were also *tcpF* positive, whereas isolates 202 and 305, which were negative for *cpe*, were also negative for *tcpF* (Fig. 3).

DISCUSSION

CPE-producing *C. perfringens* has been identified as a major cause of not only food poisoning but also non-foodborne gastrointestinal diseases including sporadic diarrhoea [8, 23], antibiotic-associated diarrhoea [9, 10] and diarrhoea in geriatric wards [10–14]. In 1993, our geriatric hospital experienced a large non-foodborne outbreak of gastroenteritis affecting 38 patients [13]. Since then, a limited number of *C. perfringens* isolation has been recorded. In 2006, increased numbers of diarrhoea cases were observed in two wards at different periods, and *cpe*-positive *C. perfringens* isolates were obtained from these cases. Although only one colony was examined for each culture plate, the positive rates of the *cpe* gene in these isolates were 71·4% and 68·8% in ward W10 and ward W08 isolates, respectively. These rates seem considerably higher than the reported positive rate of the *cpe* gene (about 5%) in human *C. perfringens* isolates [24, 25]. In addition, no enteropathogenic bacteria other than *cpe*-positive *C. perfringens* were isolated from patients in these wards. These observations lead us to suspect that the diarrhoea clusters should correspond to outbreaks that were caused by enterotoxigenic *C. perfringens*.

The chromosomal *Sma*I PFGE patterns of the outbreak isolates were mostly identical but differed in regard to four bands (Fig. 1*a*, see above). In addition, their small-plasmid profiles also differed (data not shown). The common feature is the 75-kb *cpe*-positive plasmid. These observations suggested that these outbreak isolates had been derived from the same strain, but had acquired different small plasmids in the hospital.

A plasmid carrying the IS1470-like-associated *cpe* has been shown to possess the *tcp* locus [18], and it can actually transfer by conjugation [26]. Another plasmid possessing the IS1151-associated *cpe* shares very conserved *tcp* sequences with the plasmid possessing IS1470-like-associated *cpe* [18] and with the conjugative plasmid pCW3 [19], suggesting that the plasmid harbouring the IS1151-associated *cpe* is also conjugative [18]. The 75-kb plasmids in our isolates carry the IS1151-associated *cpe* gene and the *tcp* locus, suggesting that this plasmid could have horizontally spread via conjugation to various *C. perfringens* isolates to cause nosocomial and sporadic infection. However, the site and time of the transfer are unclear. Comparative studies between the 1993 outbreak isolates and the 2006 outbreak/sporadic isolates in our hospital are now underway.

The *cpe* gene on the chromosome is reported to be associated with food poisoning, whereas this gene on plasmids is associated with non-foodborne gastrointestinal diseases [21, 27]. Accordingly, our *C. perfringens* isolates, containing the *cpe* gene on the plasmids, caused nosocomial outbreaks of non-foodborne diseases in our geriatric hospital. However, *C. perfringens* isolates having the *cpe* gene on the plasmid have previously caused foodborne outbreaks in Japan [28, 29] and in Europe [30]. In order to clarify the pathogenicity of *C. perfringens* isolates carrying the *cpe* gene on plasmids, extended epidemiological and molecular studies are required.

ACKNOWLEDGEMENTS

This work was supported by a grant (H18-Shinkou-11 from the Ministry of Health, Labour and Welfare, Japan) to A. Wada. We sincerely thank Satoko Matsunaga for her excellent technical assistance.

DECLARATION OF INTEREST

None.

REFERENCES

1. **Rood JI, Cole ST.** Molecular genetics and pathogenesis of *Clostridium perfringens*. *Microbiological Reviews* 1991; **55**: 621–648.

2. **Hauschild AHW, Hilsheimer R.** Purification and characteristics of the enterotoxin of *Clostridium perfringens* type A. *Canadian Journal of Microbiology* 1971; **17**: 1425–1433.
3. **Stark RL, Duncan CL.** Biological characteristics of *Clostridium perfringens* type A enterotoxin. *Infection and Immunity* 1971; **4**: 89–96.
4. **Stark RL, Duncan CL.** Purification and biochemical properties of *Clostridium perfringens* type A enterotoxin. *Infection and Immunity* 1972; **6**: 662–673.
5. **Sarker MR, Carman RJ, McClane BA.** Inactivation of the gene (*cpe*) encoding *Clostridium perfringens* enterotoxin eliminates the ability of two *cpe*-positive *C. perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops. *Molecular Microbiology* 1999; **33**: 946–958.
6. **Duncan CL, Strong DH.** Improved medium for sporulation of *Clostridium perfringens*. *Applied Microbiology* 1968; **16**: 82–89.
7. **Smith WP, McDonel JL.** *Clostridium perfringens* type A: in vitro system for sporulation and enterotoxin synthesis. *Journal of Bacteriology* 1980; **144**: 306–311.
8. **Brett MM, *et al.*** Detection of *Clostridium perfringens* and its enterotoxin in cases of sporadic diarrhoea. *Jounal of Clinical Pathology* 1992; **45**: 609–611.
9. **Borriello SP, *et al.*** Enterotoxigenic *Clostridium perfringens*: a possible cause of antibiotic-associated diarrhoea. *Lancet* 1984; **i**: 305–307.
10. **Borriello SP, *et al.*** Epidemiology of diarrhoea caused by enterotoxigenic *Clostridium perfringens*. *Journal of Medical Microbiology* 1985; **20**: 363–372.
11. **Williams R, *et al.*** Diarrhoea due to enterotoxigenic *Clostridium perfringens*: clinical features and management of a cluster of ten cases. *Age and Ageing* 1985; **14**: 296–302.
12. **Jackson SG, *et al.*** Diagnostic importance of *Clostridium perfringens* enterotoxin analysis in recurring enteritis among elderly, chronic care psychiatric patients. *Journal of Clinical Microbiology* 1986; **23**: 748–751.
13. **Wada A, *et al.*** Nosocomial diarrhoea in the elderly due to enterotoxigenic *Clostridium perfringens*. *Microbiology and Immunology* 1996; **40**: 767–771.
14. **Fukao T, *et al.*** An outbreak of diarrheal disease caused by enterotoxigenic *Clostridium perfringens* following exposure to a contaminated enviroment in a nursing home [in Japanese]. *Kansenshogaku Zasshi* 2004; **78**: 32–39.
15. **Yamagishi T, *et al.*** Persistent high numbers of *Clostridium perfringens* in the intestines of Japanese aged adults. *Japanese Journal of Microbiology* 1976; **20**: 397–403.
16. **Yoo HA, *et al.*** Molecular typing and epidemiological survey of prevalence of *Clostridium perfringens* types by multiplex PCR. *Journal of Clinical Microbiology* 1997; **35**: 228–232.
17. **Miyamoto K, Wen Q, McClane BA.** Multiplex PCR genotyping assay that distinguishes between isolates of *Clostridium perfringens* type A carrying a chromosomal enterotoxin gene (*cpe*) locus, a plasmid *cpe* locus with an IS*1470*-like sequence, or a plasmid *cpe* locus with an IS*1151* sequence. *Journal of Clinical Microbiology* 2004; **42**: 1552–1558.
18. **Miyamoto K, *et al.*** Complete sequencing and diversity analysis of the enterotoxin-encoding plasmids in *Clostridium perfringens* type A non-food-borne human gastrointestinal disease isolates. *Journal of Bacteriology* 2006; **188**: 1585–1598.
19. **Bannam TL, *et al.*** Functional identification of conjugation and replication regions of the tetracycline resistance plasmid pCW3 from *Clostridium perfringens*. *Journal of Bacteriology* 2006; **188**: 4942–4951.
20. **Corkill JE, *et al.*** Pulsed-field gel electrophoresis of degradation-sensitive DNAs from *Clostridium difficile* PCR ribotype 1 strains. *Journal of Clinical Microbiology* 2000; **38**: 2791–2792.
21. **Cornillot E, *et al.*** The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. *Molecular Microbiology* 1995; **15**: 639–647.
22. **Miyamoto K, *et al.*** Organization of the plasmid *cpe* locus in *Clostridium perfringens* type A isolates. *Infection and Immunity* 2002; **70**: 4261–4272. [Author's correction. 2003; **71**: 1611.]
23. **Mpamugo O, Donovan T, Brett MM.** Enterotoxigenic *Clostridium perfringens* as a cause of sporadic cases of diarrhoea. *Journal of Medical Microbiology* 1995; **43**: 442–445.
24. **Kokai-Kun JF, *et al.*** Comparison of western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. *Journal of Clinical Microbiology* 1994; **32**: 2533–2539.
25. **Daube G, *et al.*** Hybridization of 2,659 *Clostridium perfringens* isolates with gene probes for seven toxins (α, β, ε, ι, θ, μ, and enterotoxin) and for sialidase. *American Journal of Veterinary Research* 1996; **57**: 496–501.
26. **Brynestad S, *et al.*** Enterotoxin plasmid from *Clostridium perfringens* is conjugative. *Infection and Immunity* 2001; **69**: 3483–3487.
27. **Collie RE, McClane BA.** Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. *Journal of Clinical Microbiology* 1998; **36**: 30–36.
28. **Tanaka D, *et al.*** An outbreak of food-borne gastroenteritis caused by *Clostridium perfringens* carrying the *cpe* gene on a plasmid. *Japanese Journal of Infectious Diseases* 2003; **56**: 137–139.
29. **Tanaka D, *et al.*** Genotyping of *Clostridium perfringens* isolates collected from food poisoning outbreaks and healthy individuals in Japan based on *cpe* locus. *Japanese Journal of Infectious Diseases* 2007; **60**: 68–69.
30. **Lahti P, *et al.*** *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene (genotype IS*1151*-*cpe* or IS*1470*-like-*cpe*) as a common cause of food poisoning. *Journal of Clinical Microbiology* 2008; **46**: 371–373.