Multicentre study of the prevalence of toxigenic Clostridium difficile in Korea: results of a retrospective study 2000–2005

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The prevalence of toxigenic Clostridium difficile in Korea has been reported to be approximately 60–80%. Although the prevalence of the tcdA\(^-\)tcdB\(^+\) C. difficile strain was less than 5% prior to the year 2000, it has become an emerging nosocomial pathogen in Korea. Therefore, we have attempted to determine the multicentre nationwide prevalence of tcdA\(^+\)tcdB\(^+\) and tcdA\(^-\)tcdB\(^+\) C. difficile for epidemiological purposes. C. difficile strains (n=724, 30 from 2000, 80 from 2001, 74 from 2002, 76 from 2003, 179 from 2004, 285 from 2005) were obtained retrospectively from January 2000 to December 2005 from in-patients at 6 hospitals, all of whom were suspected of having C. difficile-associated disease (CDAD), colitis or pseudomembranous colitis. The numbers of participating hospitals varied yearly (1 in 2000, 2 in 2001–2003, 3 in 2004, 5 in 2005). The hospitals were located in Seoul (n=4), Kyunggi Province (n=1) and Busan (n=1), Korea. PCR assays for tcdA and tcdB genes were conducted using 724 unduplicated C. difficile isolates. The mean prevalence of tcdA\(^+\)tcdB\(^+\) and tcdA\(^-\)tcdB\(^+\) C. difficile strains over the 6 years was 51.8% (38.4–59.3%) and 25.8% (10–56.0%), respectively. The mean prevalence of tcdA\(^-\)tcdB\(^+\) C. difficile strains was less than 7% until 2002, but began to increase in 2003 (13.2%) and achieved a peak in 2004 (51.8%) (38.4–59.3%) and 25.8% (10–56.0%), respectively. This nationwide epidemiological study showed that tcdA\(^-\)tcdB\(^+\) C. difficile strains have already spread extensively throughout Korea, and our results provide basic data regarding the controversies currently surrounding the toxigenicity of tcdA\(^-\)tcdB\(^+\) C. difficile. The use of enzyme immunoassays capable of detecting both TcdA and TcdB is strongly recommended for the diagnosis of CDAD in microbiology laboratories, in order to control the spread of the tcdA\(^-\)tcdB\(^+\) strains of C. difficile.

INTRODUCTION

Clostridium difficile is one of the most common nosocomial pathogens and is responsible for C. difficile-associated disease (CDAD) and pseudomembranous colitis (PMC). C. difficile can be either toxigenic or non-toxigenic. Toxigenic C. difficile strains generally produce an enterotoxin (TcdA) and a cytotoxin (TcdB). These toxins are encoded by two genes, tcdA and tcdB, which have been mapped to a 19.6 kb chromosomal pathogenicity locus (PaLoc) (Rupnik et al., 1998). Besides both of these genes, three additional

Abbreviations: CDAD, Clostridium difficile-associated disease; EIA, enzyme immunoassay; PaLoc, pathogenicity locus; PMC, pseudomembranous colitis.
regulatory genes (tcdC, tcdD and tcdE) are located within the PaLoc (Braun et al., 1996; Hammond & Johnson, 1995; Cohen et al., 2000). However, a number of different genetic variants of C. difficile have been reported with increasing frequency worldwide. C. difficile strains with various genetic modifications within the PaLoc have been studied in the past, and 28 different toxinotypes have been identified (Rupnik et al., 1998). These include variants of C. difficile that harbour deletions, insertions or polymorphic restriction sites in one or more of the genes within the PaLoc, but still generate functional TcdA and TcdB toxins (TcdA\(^+\)TcdB\(^+\) strains). However, strains of toxinotypes VIII, X, XVI and XVII generate a functional TcdB but no TcdA (TcdA\(^-\)TcdB\(^+\) strains), whereas strains grouped into toxinotype XI harbour only the TcdA portion harbour only the TcdA\(^+\)TcdB\(^-\) portion. These strains are classified as TcdB positives (TcdB\(^+\)) and generate neither TcdA nor TcdB (TcdA\(^-\)TcdB\(^-\) strains) (Von Eichel-Streiber et al., 2000; Limaye et al., 2006; Johnson et al., 2001; Kuijper et al., 2001; Pituch et al., 2001).

The prevalence rates of these TcdA\(^-\)TcdB\(^+\) strains were reported as ranging from 0.2 to 56% in different studies from the USA, Europe and Asia (Kato et al., 1998; Lyerly et al., 1998; Pituch et al., 2001; Barbut et al., 2002; Samra et al., 2002; Rupnik et al., 2003; Geric et al., 2004). However, as the TcdA\(^-\)TcdB\(^+\) C. difficile prevalence rate has been reported to be as high as 39% in one Japanese study (Komatsu et al., 2003) and a highly prevalent TcdA\(^-\)TcdB\(^+\) C. difficile strain was detected in a tertiary hospital in Korea, this variant strain has become recognized as an emerging pathogen in Korea (Shin & Kuak, 2006; Shin et al., 2008). Therefore, it is important to determine at what time the TcdA\(^-\)TcdB\(^+\) C. difficile strains became prevalent and when they really began to be widely distributed throughout Korea. In this study, we have investigated the nationwide prevalence of TcdA\(^+\)TcdB\(^+\) and TcdA\(^-\)TcdB\(^+\) C. difficile strains in Korea from 2000 to 2005 for epidemiological purposes.

**METHODS**

**Specimens.** A total of 724 strains of C. difficile (30 from 2000, 80 from 2001, 74 from 2002, 76 from 2003, 179 from 2004, 285 from 2006) were obtained between January 2000 and December 2005 from in-patients at 6 hospitals suspected of harbouring CDAD, colitis or PMC. The numbers of participating hospitals varied yearly (1 in 2000, 2 in 2001–2003, 3 in 2004, 5 in 2005). The hospitals were located in Seoul (n=4), Kyunggi Province (n=1) and Busan (n=1), Korea, and the mean number of beds was 920, with a range of 400–2000 (Fig. 1).

**C. difficile cultures.** Stool samples collected from patients suspected to be infected with C. difficile were inoculated into anaerobically reduced cycloserine–cefoxitin–fructose agar at 37 °C under anaerobic conditions for 48–72 h in each participating hospital. The identification of C. difficile was conducted in each participating laboratory in accordance with local techniques, which included analysis of Gram stain, spore stain, characteristic odour and typical morphological features, and/or enzyme immunoassays (EIAs) to detect TcdA. Isolates sent to the central coordinating laboratory were then subcultured, and identification was confirmed via a biochemical assay using an ANA identification test kit (bioMérieux).

**PCR assay for tcdA and tcdB.** Genes that encode the large clostridial toxins A and B (tcdA and tcdB) were detected by PCR in accordance with the methods described by Kato et al. (1998), with some modifications, on 724 strains of C. difficile. Template DNA was prepared by suspending 20 colonies in a 5% (w/v) solution of Chelex-100 (Bio-Rad), boiling for 12 min, then centrifuging for 5 min at 12 000 g. The total volume of the PCR reaction was 100 μl, and it contained ~30 ng bacterial DNA preparation, 0.15 μg each primer, the four dNTPs (200 μM each), 10 mM Tris/HCl (pH 8.3), 2.5 mM MgCl\(_2\), 50 mM KCl and 2.5 U Taq polymerase. tcdA and tcdB were amplified using the following primers: NK9 (5’-GCACGCTGCAGGATAAC-3’) and NK11 (5’-TGATGCTAATAATGAA TCTAAATGGTGTAAC-3’), which were derived from the repeating sequence of tcdA; and primers NK104 (5’-GGTGTAGCATGAAA GTCCAATTTACGC-3’) and NK105 (5’-CATTATGCTTCTT GATTGTCGACCT-3’), which were derived from the non-repeating sequence of tcdB. The ATCC 43596 strain (serogroup C) was utilized as the tcdA\(^+\)tcdB\(^+\) control and ATCC 43598 strain (serogroup F) was used as the tcdA\(^-\)tcdB\(^+\) variant control. PCR amplification using NK9/NK11 was conducted in a thermal cycler (Perkin-Elmer) with 40 cycles of 95 °C for 15 s, 62 °C for 120 s and 72 °C for 40 s. For the primer pairs NK104/NK105 as the following thermal profile was used: 40 cycles at 95 °C for 20 s, 62 °C for 60 s and 74 °C for 40 s. At the conclusion of these PCR cycles, the tubes were incubated for 5 min at 74 °C. Following completion of the PCRs, 10 μl amplified product was electrophoresed in 2% agarose gel and the bands were visualized by UV transillumination.

Strains in which the tcdA gene was intact yielded 1200 bp PCR products, and tcdA\(^-\)tcdB\(^+\) variant strains, yielded 700 or 500 bp PCR products. In C. difficile strains with an intact tcdB\(^+\) gene, the PCR product for tcdB was 204 bp.
RESULTS

The total number of C. difficile isolates studied was 724. As we conducted the PCR assay for tcdA and tcdB retrospectively, the number of strains available was only 30 in 2000, but the number was increased to 285 in 2005. The prevalences of tcdA⁺tcdB⁺ and tcdA⁻tcdB⁺ C. difficile strains were 93.3 and 6.7% in 2000. No tcdA⁻tcdB⁻ C. difficile strain was detected in that year. However, the prevalence of the tcdA⁻tcdB⁺ strain showed a tendency toward decrease from 2001 (73.8%) and fell to its lowest level in 2004, as low as 35.2%. In 2005, the mean prevalence of tcdA⁺tcdB⁺ C. difficile was 47.7%, ranging from 30.9 to 60.3%. The prevalence of the tcdA⁻tcdB⁺ strain began to increase, up to 13.2% in 2003, and finally up to 50.3% in 2004. In 2005, the mean prevalence of the tcdA⁻tcdB⁺ strains was 27.0%, ranging from 17.6 to 54.8%. The tcdA⁻tcdB⁺ variant strains appeared to become endemic nationwide beginning in 2005. The mean prevalence of tcdA⁺tcdB⁻ C. difficile strains was approximately 22.4% for 6 years, ranging from 0% (2000) to 32.9% (2003).

Although the mean prevalences of the tcdA⁺tcdB⁺ and tcdA⁻tcdB⁺ C. difficile strains over the 6 years were 51.8% (range 33.3–69.9%) and 25.8% (range 10–56%), the distributions of tcdA⁺tcdB⁺ and tcdA⁻tcdB⁺ C. difficile strains differed from hospital to hospital and from year to year (Fig. 2). In 2000, the C. difficile strains were collected only from one hospital (hospital A) and the prevalence of the variant strain in that hospital was 6.7%. In 2001, isolates of C. difficile were acquired from two hospitals (hospitals A and B). However, the variant strains were not observed in the participating hospital in 2000, although the mean prevalence of the variant strain was 5%. In 2002, the rate of prevalence of the variant strain was 4.2% (hospital A) and 6.0% (hospital B), respectively. In 2003, the prevalence of the variant strains began to increase up to 16.7% in one hospital, whereas it remained 7.1% in the other participating hospital. In 2004, another two hospitals newly participated in the surveillance (hospitals C and D), and one hospital (hospital B) did not participate in the follow-up. One of them (hospital D) reported that the prevalence of tcdA⁺tcdB⁺ C. difficile remained less than 7% (6.4%), but the other hospital (hospital C) reported highly prevalent tcdA⁺tcdB⁺ C. difficile strains (71.8%), suggesting an outbreak. The other continuing participating hospital (hospital A) reported an increased prevalence of tcdA⁻tcdB⁺ strains (36.4%), which was also suggestive of outbreak.

In 2005, another two hospitals (hospitals E and F) joined the surveillance, and they already had highly prevalent tcdA⁺tcdB⁺ strains (25.6 and 54.8%, respectively). Their tcdA⁺tcdB⁺ and tcdA⁻tcdB⁻ C. difficile rates were 53.9/20.5% (hospital E) and 30.9%/14.3% (hospital F), respectively.

DISCUSSION

TcdA⁻TcdB⁺ C. difficile has been reported in several countries, with varying prevalence rates. As highly prevalent tcdA⁻ variant strains were previously isolated from patients with CDAD in a tertiary hospital in Korea (Shin & Kuak, 2006; Shin et al., 2008), further nationwide surveillance was required in order to sufficiently ascertain the prevalence and virulence of tcdA⁺tcdB⁺ strains of C. difficile in Korea. As published studies revealed no tcdA⁻tcdB⁺ strains among C. difficile isolates in Korea (Kato et al., 1998; Rupnik et al., 2003) or lower prevalence rates (4.3%) of these strains (Chung et al., 2002), we needed to determine at what time these C. difficile strains became prevalent and truly widely spread throughout Korea. This study is believed to be the first nationwide surveillance of C. difficile isolates from patients suspected of having CDAD in Korea.

We retrospectively obtained 724 C. difficile strains stored in each of 6 hospitals over 6 years, from 2000 to 2005. The mean prevalence of tcdA⁻tcdB⁻ strains was less than 10% (range 5.5–6.7%) until 2002, but it almost doubled (13.2%) in 2003 and reached a maximum at 2004, with levels as high as 50.3%. The mean prevalence of tcdA⁻tcdB⁺ strains in 2005 was 27.0% (range 17.6–54.8%), which was approximately four times higher than was reported in 2000.

The prevalence rates of TcdA⁻TcdB⁺ strains were reported to be 1.3–2% in the USA (Lyerly et al., 1998; Geric et al., 2004) and 2.7% in France (Barbut et al., 2002). In the UK, it was estimated that approximately 3% of toxigenic C. difficile are TcdA⁻TcdB⁺ strains (Brazier et al., 1999). It was identified in 11% of CDAD cases in Poland (Pituch et al., 2001). A recent 2 month prospective study in Europe in 2005 showed that 24.3% of C. difficile isolates were toxin...
variants, and the prevalence rate of toxinotype VIII was 5% overall among them (Barbut et al., 2007). By way of contrast, they reported that PCR ribotype 017 was particularly predominant in Poland, Ireland, Greece and Sweden, and all PCR ribotype 017 isolates belonged to toxinotype VIII and were tcdA−tcdB+ strains. Other recent studies reported that tcdA−tcdB+ strains accounted for 56% in Israel and 44% in Ireland (Samra et al., 2002; Drudy et al., 2007).

As compared with these reports, the prevalence of tcdA−tcdB+ strains in Korea was less than or similar to that of the other countries until 2002–2003, but has increased since 2004. Our results revealed that outbreaks of tcdA−tcdB+ strains were experienced in two hospitals in 2004; infection with these strains remained highly prevalent in 2005, as compared with published reports in Korea (Shin & Kim, 1992; Lee & Chung, 1993; Lee et al., 1999; Kang et al., 2000; Chung et al., 2002). One hospital (hospital F), which was involved only in 2005, is located 450 km from other participating hospitals. However, the prevalence of tcdA−tcdB+ strains in that hospital had already reached a level of 56%. The prevalence of variant strains in Japan was reported to be 12.5% (Kato et al., 1998). However, variant prevalence rates as high as 39% during a 1 year period (December 1999–November 2000) have been reported in CDAD patients, although this was a report issued by a Japanese hospital (Komatsu et al., 2003). These results suggested that tcdA−tcdB+ strains had spread throughout Far East Asia, including Korea and Japan, before 2000, and these tcdA−tcdB+ strains appeared to be endemic in many Korean hospitals since 2004.

We are not currently precisely certain as to why we had such highly prevalent tcdA−tcdB+ strains in Korea over such a brief duration of time. We surmised that one reason might be that patients are constantly on the move seeking better medical service, and C. difficile is a pathogen that is quite difficult to eradicate, because of its ability to sporulate (McFarland et al., 1989). Another factor may be a failure of infection control due to the lack of awareness of the physicians involved. It appears likely that, at some point, there must have been a clinical sign of a C. difficile variant. However, we may have missed this because we did not possess adequate data regarding the nationwide prevalence of C. difficile variants prior to 2005, because culturing, C. difficile is a strenuous procedure in the majority of laboratories, and ELAs for TcdA only have been widely utilized for the diagnosis of C. difficile infection in Korea, as has been reported in other European countries (Barbut et al., 2003). These commercial toxin A ELAs are not capable of detecting tcdA−tcdB+ variants, as variant strains of C. difficile that harbour deletions of 1.7–1.8 kb in tcdA yielded no detectable TcdA (Kato et al., 1998; Von Eichel-Streiber et al., 1999; Rupnik et al., 1997). Therefore, no attention appeared to be paid previously to infection control, especially with regard to tcdA−tcdB+ C. difficile strains. Another factor to be considered is that the diagnostic algorithms of CDAD differ from hospital to hospital. Therefore, these outbreaks appear to be associated with delays in CDAD diagnosis in certain hospitals. All of these factors may result in a significant burden on the health-care system associated with CDAD (Riley et al., 1995).

In conclusion, this nationwide epidemiological study showed that tcdA tcdB+ C. difficile strains have already spread widely in Korea, and ELAs capable of detecting both TcdA and TcdB should be utilized in microbiology laboratories in order to control these strains of C. difficile in Korea.

REFERENCES


Prevalence of toxigenic \textit{C. difficile} in Korea


