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SHORT REPORT
Rapid spread of *Clostridium difficile* NAP1/027/ST1 in Chile confirms the emergence of the epidemic strain in Latin America

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SUMMARY

Clostridium difficile infection has gained importance in recent years as a result of the rapid spread of epidemic strains, including hypervirulent strains. This study reports the molecular epidemiology of *C. difficile* obtained from hospitalized patients in Chile. Seven hundred and nineteen isolates of toxigenic *C. difficile* from 45 hospitals across the country were characterized through toxin profile, pulsed-field gel electrophoresis (PFGE), and sequencing of the *tcdC* gene. In addition, polymerase chain reaction (PCR) ribotyping and multilocus sequence typing (MLST) were performed on a subset of selected strains. PFGE typing of 719 isolates of *C. difficile* produced 60 PFGE patterns (subtypes). Subtype 1 was predominant (79% of isolates) and related to the hypervirulent strain (NAP1). Subtype 1 showed 73% relatedness with nine other subtypes, which had a similar *tcdC* deletion. Subtype 1 corresponded to ribotype 027 and ST1. This report shows the wide dissemination of the hypervirulent strain NAP1/027/ST1 in Chile.

Key words: *Clostridium difficile*, MLST, PFGE, ribotype.

Clostridium difficile, the causative agent of antibiotic-associated diarrhoea and pseudomembranous colitis, is currently one of the most important nosocomial pathogens. The main virulence factor of *C. difficile* are the two large clostridial glucosylating toxins, toxin A (TcdA) and toxin B (TcdB), which have enterotoxic and cytotoxic activity, respectively. These genes are located within a pathogenicity locus (PaLoc) [1]. The expression of the toxin genes is induced by the positive regulator TcdR and repressed by TcdC. Deletions in the *tcd*C gene from various isolates have been reported, including a common 18-bp deletion and a 1-bp deletion at nucleotide 117, leading to the expression of a truncated TcdC [2].

The rate and severity of nosocomial infections increased between 2000 and 2008, coincident with the emergence of a hypervirulent fluoroquinolone-resistant clone, designated polymerase chain reaction (PCR) ribotype 027. Outbreaks of *C. difficile* infection caused by the 027 clone have been reported in North America and throughout Europe. However, from 2007 in European countries with implementation of incidence monitoring of *C. difficile* infection, the number of cases has either remained relatively static or there has been a substantial reduction in the overall number of cases, due probably to more effective prevention measures [3]. Although incidence of

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C. difficile infection in North America and Europe is well documented, little is known about the spread of this disease in Latin America. Recently, detection of isolates of the 027/BI/NAP1 strain from patients with *C. difficile*-associated disease (CDAD) has been described in some countries of Latin America, e.g. Costa Rica, Panama and Chile [4–6]. Quesada-Gómez *et al.* [5] reported that epidemic *C. difficile* 027 strains are present in South America, highlighting the need for enhanced screening for this ribotype in other regions of the continent [7].

Multiple techniques have been used to study the epidemiology of *C. difficile* infections including pulsed-field gel electrophoresis (PFGE), restriction endonuclease analysis of total DNA, PCR ribotyping, multilocus sequence typing (MLST) and multilocus variable-number tandem repeat assays [8, 9].

This study reports the spread of *C. difficile* due to the NAP1/027/ST1 strain in different Chilean hospitals, with the aim of contributing to a better knowledge of the epidemiology of this agent in Latin America.

From January 2012 to December 2013, 45 hospitals from different part of Chile sent 1353 stool samples from patients with nosocomial CDAD to the National Laboratory of the Public Health Institute of Chile. Of the 45 hospitals, four were from the northern zone, 34 were from the central zone and seven were from the southern zone. Geographical data were analysed using epidemiological week, which referred to CDC week. Isolation and identification of *C. difficile* were performed as described previously [10]. Antimicrobial susceptibility testing for metronidazole and vancomycin was performed using the agar dilution method recommended by the Clinical Laboratory Standards Institute and Eucast (http//www.eucast.org). Quality control was performed using *Bacteroides fragilis* (ATCC 25285), *B. thetaiotaomicron* (ATCC 29741) and *C. difficile* (ATCC 700057) isolates. PFGE was performed as described by Miller *et al.* [11] with the following modifications. Genomic DNA was digested with 35 U *Sma*I overnight and a Bio-Rad PFGE system was used for electrophoresis. Dendrograms were constructed using BioNumerics software v. 6.6 (Applied Maths, Belgium) by the UPGMA clustering method using the Dice coefficient with both position tolerance and optimization of 1·10%. For the other molecular characterizations, genomic DNA was extracted from the isolates using the Qiagen DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer's instructions for Gram-positive organisms and stored at −20 °C for further study. The *tcd*A and *tcd*B toxin genes were detected by PCR as described by Lemee *et al.* [12]. The *tcd*C gene was amplified by PCR using primers C1 and C2 as described previously [2]. MLST was performed using the previously described scheme of Griffiths *et al.* [8]. Amplicons of *tcd*C and MLST housekeeping genes were purified and sequenced on an ABI 3500 DNA sequencer (Applied Biosystems, USA). DNA sequences were submitted to the MLST database (http://pubmlst.org/cdifficile) to obtain the sequence type (ST) [8]. Capillary gel electrophoresis-based PCR ribotyping was performed as described previously by Indra *et al.* [13]. The size of each peak was determined using GeneMapper software v. 4.0 (Applied Biosystems) and using a web-based software program (http://webribo.ages.at).

Of the 1353 stool samples cultured, *C. difficile* isolates were obtained from 943 (69·7%). Of these 943 *C. difficile* isolates, 719 were characterized by PFGE and *tcd*C gene sequencing (392 isolates from 2012 and 327 isolates from 2013). Of these isolates, 716 were positive for both toxins A and B (A^+B^+) and three were toxin A-negative toxin B-positive (A^-B^+). The 719 isolates analysed in the present study corresponded to 664 adult hospitalized patients (age 18–64 years, $n = 248$; $\geqslant 65$ years, $n = 416$) and the other 55 isolates were from hospitalized children (aged <18 years).

PFGE typing performed on 719 *C. difficile* isolates revealed 60 different PFGE patterns (hereinafter named subtype). Subtype 1 (568 isolates, 79%) and subtype 3 (24 isolates, 3·3%) were predominant. Of the 29 hospitals for which at least three isolates were typed by PFGE, 27 (93%) has at least one subtype 1 isolate. For 25 (86%) hospitals, subtype 1 was the predominant strain and 22 isolates from those hospitals were in the central zone of the country. Ten hospitals (26·6%) had at least one strain of subtype 3 which was not predominant in those hospitals. At the beginning of 2012, subtype 1 was detected in the central zone of the country, and subsequently detected in the northern zone (epidemiological week 20, 2012) and later in the southern zone (epidemiological week 30, 2012) (Supplementary Fig. S1). This result showed the spread of subtype 1 across Chile. Antibiotic susceptibility was performed on 436 *C. difficile* isolates, 339 of subtype 1, 18 of subtype 3 and 79 corresponding to other subtypes. The minimum inhibitory concentration required to inhibit growth of 50%

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(MIC_{50}) of metronidazole and vancomycin was 0·1 mg/l and 0·5 mg/l, respectively and the MIC_{90} of metronidazole and vancomycin was 0·2 mg/l and 1 mg/l, respectively. For vancomycin, one isolate (subtype 10) had reduced susceptibility (MIC of 4 mg/l).

Both 18-bp and nucleotide 117 *tcd*C deletions were found in 583 isolates (Fig. 1). A single deletion at position 117 (Δ117) introduced a stop codon at position 196, truncating the predicted TcdC to 65 amino-acid residues in all these isolates. Six isolates corresponding to three subtypes had a deletion of a more significant size. Subtypes 21 and 24 had deletions of 54 bp and 39 bp, respectively. These subtypes also had a nonsense mutation (C184T) that resulted in a severe truncation of the TcdC protein from 232 to 61 amino-acid residues. Subtype 35 had a deletion of 36 bp, and also had a nonsense mutation (C191A) that is predicted to truncate TcdC protein from 63 amino-acid residues. Subtype 34 with an 18-bp deletion also had the nonsense mutation C184T. Thirteen subtypes with the truncated *tcd*C gene were found in this study and only subtype 1 was predominant. All of these mutations have been previously described by Curry *et al.* [2]. It has been described that strains with an aberrant *tcd*C genotype increase toxin production and hence increase virulence [2]. The notion that strains of *C. difficile* that produce more toxins are intrinsically more virulent is debatable. However, Cartman *et al.* [14] has demonstrated through deletion and restoration of *tcd*C that there is no association between the *tcd*C genotype and toxin production.

On the basis of PFGE patterns and *tcd*C deletions, a major cluster was identified (Fig. 1). This cluster included 10 subtypes showing 73% relatedness, which exhibited the PFGE pattern of the NAP1 strain [3, 5]. Subtype 1 accounted for 97% of the isolates of this cluster. In order to confirm that isolates of this cluster correspond to the NAP1 strain, a subset of *C. difficile* isolates were analysed by PCR ribotyping and MLST. A total of 250 of the 719 isolates were tested by PCR ribotyping. Of these, 176 isolates were selected from the major cluster (160 subtype 1 isolates were selected from 37 hospitals and 16 isolates were from other subtypes of the major cluster) and 74 isolates were randomly selected from the remaining subtypes. Overall, 31 ribotypes were identified of which 18 were new ribotypes. Isolates from the 10 subtypes of the major cluster corresponded to ribotype 027, including subtype 1 which was predominant in this study. The use of the capillary gel electrophoresis-based PCR ribotyping method supported by a web-based database could resolve both the problems associated with assignment of ribotypes and comparison of typing results between laboratories [13]. One hundred isolates were analysed by MLST, 90 isolates were selected from the major cluster (81 subtype 1 isolates were selected from 37 hospitals and nine isolates were from other subtypes of the major cluster). Moreover, 10 randomly selected isolates from the remaining subtypes were analysed. MLST generated three different STs from the isolates of the major cluster. ST1 was found in seven subtypes, including subtype 1. The relationships between the STs were examined using the concatenated sequences of the seven MLST loci to construct a neighbour-joining tree (data not shown). ST1, ST41, and ST67 corresponded to the hypervirulent cluster described by Griffiths *et al.* [8]. The second most common PFGE pattern (subtype 3) found in this study corresponded to the PFGE pattern of the NAP2 strain. To confirm this result, three isolates were analysed by MLST. All isolates tested were ST3, which has been described as the NAP2 strain. No isolates of subtype 3 had a deletion in the *tcd*C gene. On the other hand, strains were currently increasing in other countries such as ribotype 078 and were not detected in the isolates analysed by PCR ribotyping [5, 9].

Strain NAP1/027/ST1 has been responsible for large outbreaks in healthcare facilities in North American, European, Asian and more recently in some Latin American countries. The first outbreak of the NAP1 strain in Latin America occurred in Costa Rica in 2010 and was detected in 20 hospitalized patients [4]. Later NAP1 strain was identified in six hospitalized patients in Panama in 2012 [5]. In Chile, Hernández-Rocha *et al.* [6] described ribotype 027 isolates from two hospitalized patients in 2012. The present study shows the molecular characterization of 719 isolates of *C. difficile* in different hospitals, and shows that the NAP1/027/ST1 strain has spread throughout the country. After the increase of *C. difficile* infections in hospitals in Chile, the infection control programme of the Ministry of Health of Chile strengthened the recommendations for prevention and outbreak control for *C. difficile* infections, placing the emphasis on avoiding patient contact, cleanliness of the environment and appropriate antimicrobial therapy. In addition, laboratory testing techniques for the diagnosis of *C. difficile* infections were increased in regional hospitals.

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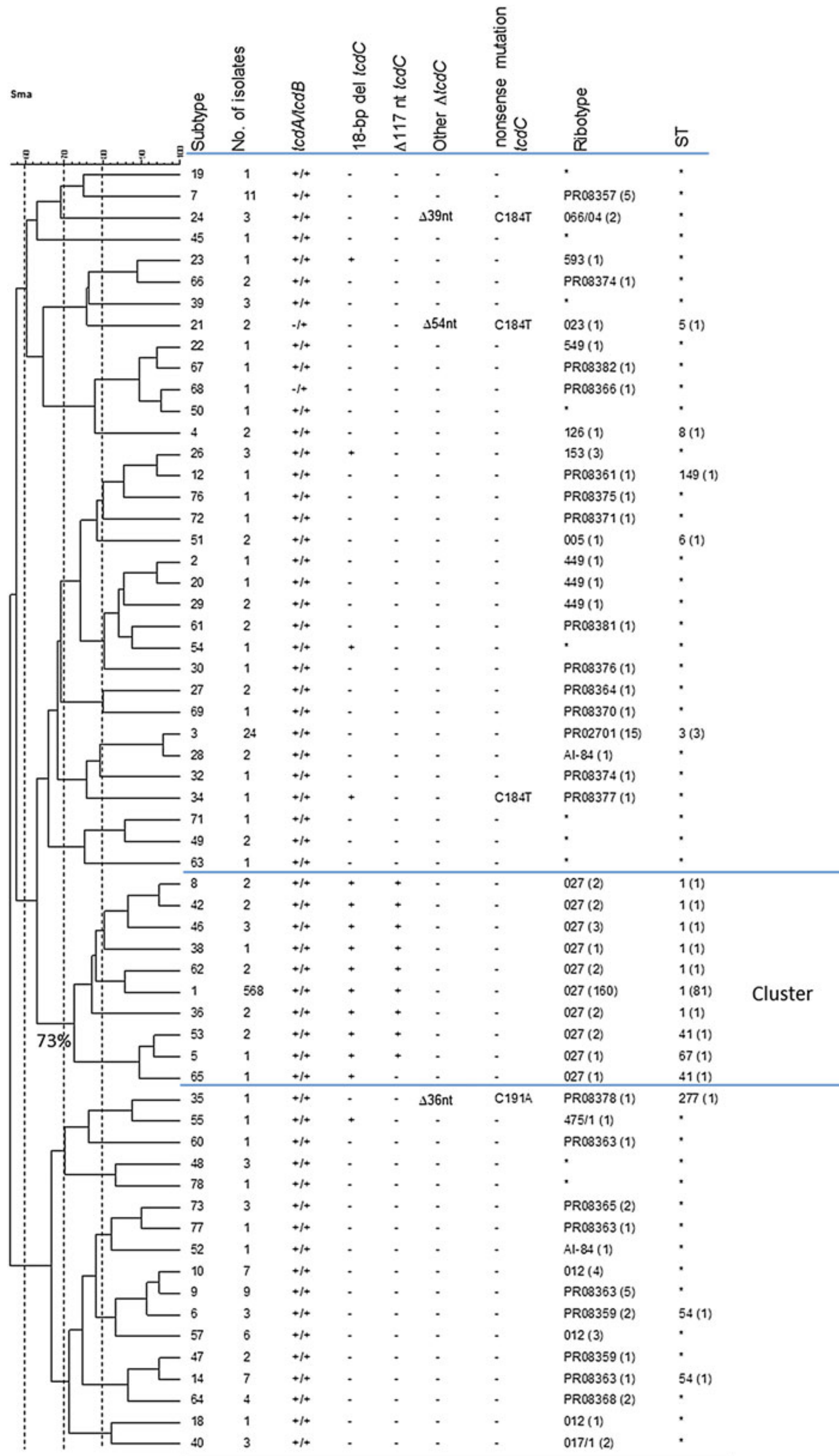

Fig. 1. Phylogenetic analysis of 60 PFGE patterns representing 719 *Clostridium difficile* isolates. +, presence; –, absence. Ribotyping and MLST: * indicates isolates not tested; numbers in parentheses are number of isolates from each type.

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Since the emergence of the *C. difficile* ribotype 027 epidemic strain in North America and Europe, this is the first report of the spread of this strain in Latin America, with an incidence of 79% of the NAP1/027 strain. Therefore, surveillance programmes for C. *difficile* infection should be implemented in public health facilities to strengthen the diagnosis and to better characterize strains that produce hospital outbreaks.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0950268815000023.

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DECLARATION OF INTEREST

None.

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