The Brazilian Journal of
INFECTIOUS DISEASES

www.elsevier.com/locate/bjid

Brief communication

A search for *Clostridium difficile* ribotypes 027 and 078 in Brazil

Alexandre de Almeida Monteiro[a], *Renata Neto Pires*[a,b], *Søren Persson*[c], *Edison Moraes Rodrigues Filho*[d], *Alessandro Comarú Pasqualotto*[a,b,*]

[a] *PPG Patologia, Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, RS, Brazil*
[b] *Santa Casa de Misericórdia de Porto Alegre, Porto Alegre, RS, Brazil*
[c] *Statens Serum Institut, Copenhagen, Denmark*
[d] *Grupo Hospitalar Conceição, Porto Alegre, RS, Brazil*

ARTICLE INFO

Article history:
Received 1 August 2014
Accepted 21 August 2014
Available online 11 October 2014

Keywords:
Multiplex-PCR
Clostridium difficile
HRM
Toxin gene profiling

ABSTRACT

Toxigenic strains of *Clostridium difficile* may be disseminating. Here we prospectively screened patients with nosocomial diarrhoea in two hospitals in Brazil. To identify *C. difficile* polymerase chain reaction ribotypes 027/078 strains, we used high resolution melting and multiplex polymerase chain reaction. Among 116 screened patients, 11 were positive for *C. difficile*. The polymerase chain reaction ribotypes 027/078 strains were not identified in this study.

Introduction

Clostridium difficile is the main infectious agent of nosocomial diarrhoea, which may result in significant morbimortality.[1] Recently, toxigenic strains of *C. difficile* have emerged, sometimes causing outbreaks.[2] Most of these infections are caused by the polymerase chain reaction (PCR) ribotypes 027 and 078 also referred to as BI/NAP1 and BK/NAP7, respectively. Very limited data are available on the frequency of toxigenic strains in Latin America. A study from Costa Rica revealed that 54% (20/37) isolates of *C. difficile* were identified as *C. difficile* PCR ribotype 027.[3] No other study has documented toxigenic *C. difficile* strains in Latin America. Here we investigated whether strains 027/078 were present in cases of nosocomial diarrhoea in a cohort study in Brazil.

This was a multicenter prospective observational study, conducted in two hospitals in Porto Alegre, Brazil.[4] Hospital 1 is a 65-bed transplant hospital; Hospital 2 is a 800-bed tertiary hospital, including 40 intensive care unit beds. Inpatient adults (>18 years-old) passing ≥3 liquid stools over a 24 h period were included. Participation in the study was conditioned to signing an informed consent (ISCMPA Ethics Committee approval – 304/010).

* *Corresponding author at*: Molecular Biology Laboratory, Santa Casa de Misericordia de Porto Alegre, Av Independência 155, 8° andar, 90035-074, Porto Alegre, Brazil.
E-mail addresses: acpasqualotto@hotmail.com, pasqualotto@ufcspa.edu.br (A.C. Pasqualotto).
http://dx.doi.org/10.1016/j.bjid.2014.08.004

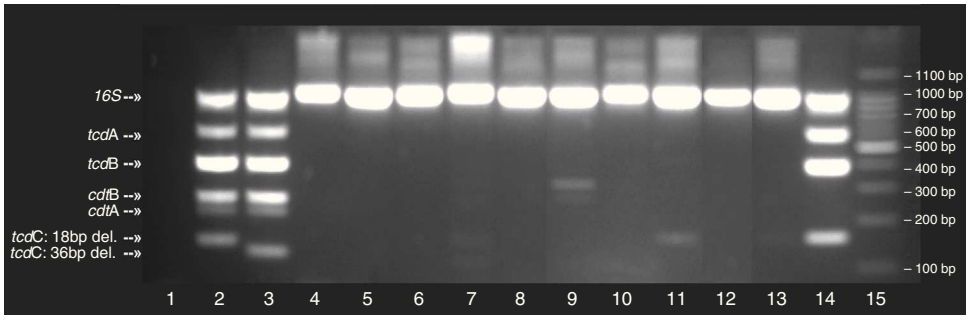

Fig. 1 – Typing of *C. difficile* using multiplex conventional polymerase chain reaction (PCR) on 11 clinical isolates. Lane 1: no template control; Lane 2: *C. difficile* 027 (positive control strain); Lane 3, *C. difficile* 078 (positive control strain); Lane 15: 100 bp DNA ladder; Additional lanes: clinical samples numbered according to their identification in the study; The position of the different *C. difficile* gene toxins (i.e., *tcdA*, *tcdB*, *cdtA*, *cdtB*, *tcdC*) as well as 16S rDNA, internal control are marked on the gel, including the deletions for *tcdC*.

Faecal samples were inoculated on *C. difficile* agar (CLO) selective medium (bioMérieux, France) and incubated anaerobically for 48 h at 37 °C. DNA was extracted from bacterial colonies using QIAamp DNA Stool Mini Kit, Qiagen. Molecular characterization of strains was performed by conventional multiplex PCR (mPCR) test, as previously published.[5] mPCRs were adjusted to a final volume of 25 μL, containing the following: 2.5 μL of 10× PCR buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 2 mM $MgCl_2$, 260 mM of each deoxyribonucleotide, 1.5 U of Platinum *Taq* DNA Polymerase (Invitrogen), primers (same primers and concentrations as used in Persson et al.[6]), and 5 μL of genomic DNA. Positive controls used in this reaction were obtained from the Statens Serum Institut – Denmark (*C. difficile* 027 – F62 and *C. difficile* 078 – M16). Milli-Q® water was used as a negative control. All mPCRs were performed in a Veriti thermal cycler (Life Technologies). Cycling conditions were: 10 min at 94 °C, followed by 35 cycles of 50 s at 94 °C, 40 s at 54 °C, 50 s at 72 °C, and a final extension of 3 min at 72 °C.

Amplicons sizes were checked by 3.0% (w/v) agarose gel-electrophoresis stained with ethidium bromide. The amplicons *tcdC* (126 and 144 bp, resulting from internal deletions of sizes 39 and 18 bp, respectively), as well as the amplicons 16S rDNA, *tcdA*, *tcdB* and *tcdA*/*tcdB*, were used as standards in each reaction.

Besides the molecular characterization of *C. difficile* by mPCR, we optimized a qPCR using high resolution melting (HRM) analysis, adapting the protocol described by Grando et al.[7] Type-it HRM PCR (Qiagen) was used to prepare the mix. The PCR mixtures were optimized for a volume of 50 μL, containing 25 μL of 2× HRM PCR Master Mix (Qiagen), 0.5 μM of both forward and reverse primers, 15 μL Milli-Q water and 5 μL of the genomic template DNA (or Milli-Q water as no template control). Thermocycling conditions and curve normalization were performed according the protocol proposed by Grando et al.[7] After normalizing the curves, results were interpreted by subtracting the melting-curve shapes generated by the standard (*C. difficile* 027 reference strain) from the curves generated for the other isolates and standards.

From the 116 patients included in the study, 11 had positive cultures for *C. difficile*, and these were evaluated for the presence of hypervirulent characteristics. Among these, 10 clinical strains were non-toxigenic while one strain harboured *tcdA* and *tcdB*. When compared to controls, none of the isolates had toxin gene profiles similar to ribotypes 027/078 (Fig. 1). These results were confirmed by HRM, in which none of the isolates showed peaks compatible with PCR ribotypes 027/078 (Fig. 2).

Molecular typing of *C. difficile* is still not widely used in tertiary hospitals in Brazil, despite the known emergence of toxigenic *C. difficile* strains. Proper typing of *C. difficile* may be of relevance for infection control purposes. Several methods for the differentiation of *C. difficile* isolates are currently available, including PCR-ribotyping, restriction endonuclease analysis, and pulsed-field gel electrophoresis.[8] Performing more than one typing method could potentially increase the discriminatory power of a single typing method, even though few laboratories follow this practice.[2] The mPCR is a rapid and specific method for screening toxin genes of *C. difficile*. This is a simple and relatively cheap method that allows for the presumptive typing of *C. difficile* strains.[6] On the other hand, HRM analysis of PCR products can identify the different genotypic variations among different bacteria. Despite being a more expensive method, HRM offers quicker results and has demonstrated a discriminatory power as high as 0.928.[7]

In this study, no patient was found to be infected by *C. difficile* PCR ribotype 027/078 strains. Similar findings were described in a previous study in Brazil, in which ribotyping was performed.[9] So far, these strains have not been documented in Brazil. However, considering that molecular typing technologies and anaerobic cultures are still limited in the country, underdetection of *C. difficile* ribotypes 027/078 strains is also a possibility.[9] Even though PCR ribotype 027/078 strains were not detected in this study, our analysis revealed the presence of nine different HRM *C. difficile* profiles (data not shown).

This study has some limitations. Unfortunately it was not possible to classify the samples that were negative for PCR ribotype 027/078, according to the nomenclature proposed by Stubbs et al.[10] Also, despite being a prospective multicenter study, only a small number of isolates tested positive for *C. difficile* (11/116). Increasing sample size could potentially result in the identification of hypervirulent *C. difficile* strains. As all isolates showed different peaks in comparison to controls, the

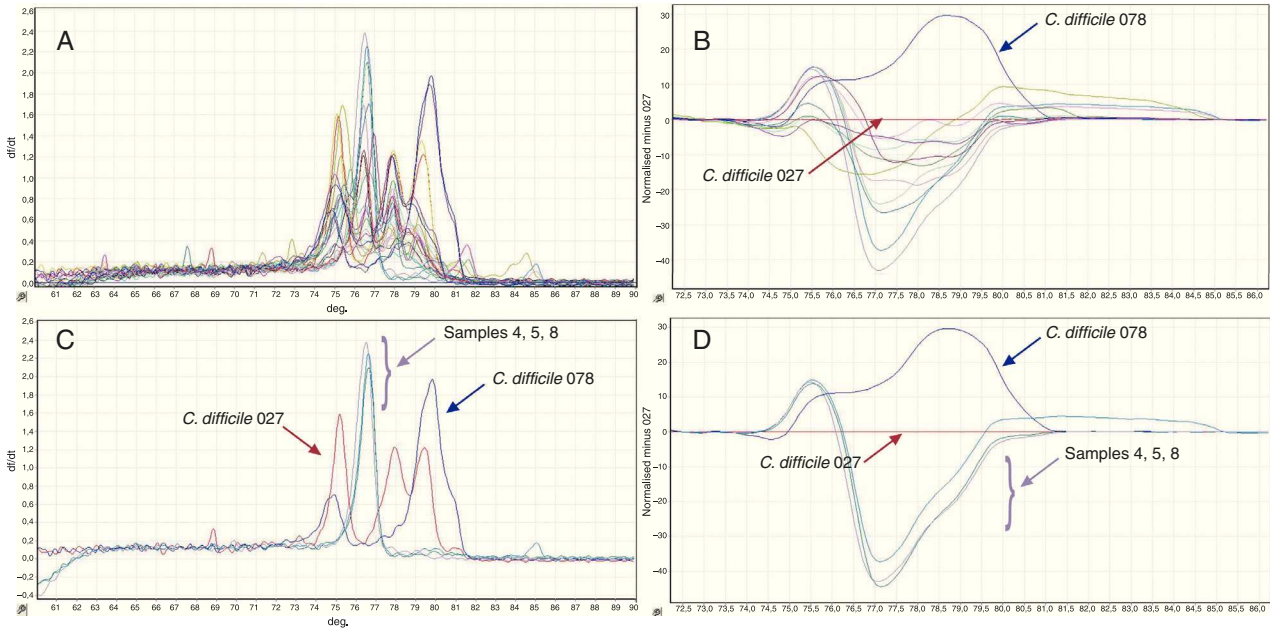

Fig. 2 – (A) Melt peak curves on the HRM analysis for the two positive control strains (*C. difficile* 027/078) and the 11 clinical isolates included in this study. (B) Two positive control strains as well as clinical isolates 4, 5 and 8. (C) Difference plotting of the HMR curves for *C. difficile* 078 positive control strain, in addition to 11 clinical isolates; *C. difficile* 027 is used as reference in the graphic. (D) Two control strains (027/078) and clinical isolates 4, 5 and 8.

discriminatory power of the methods for the detection of *C. difficile* 027/078 could not be determined.

In conclusion, this study did not detect the presence of *C. difficile* 027/078 hypervirulent strains in a prospective cohort of patients with nosocomial diarrhoea in Brazil. The importance of implementing molecular typing methods for *C. difficile* in a continental country such as Brazil deserves additional attention.

Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES

1. Pepin J, Valiquette L, Alary M, et al. *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. CMAJ. 2004;171:466–72.
2. Tenover FC, Akerlund T, Gerding DN, et al. Comparison of strain typing results for *Clostridium difficile* isolates from North America. J Clin Microbiol. 2011;49:1831–7.
3. Quesada-Gómez C, Rodríguez C, del Mar Gamboa-Coronado M, et al. Emergence of *Clostridium difficile* NAP1 in Latin America. J Clin Microbiol. 2010;48:669–70.
4. Pires RN, Monteiro AA, Carneiro LC, et al. *Clostridium difficile* infection in Brazil: a neglected problem? Am J Infect Control. 2014;42:459–60.
5. Persson S, Jensen JN, Olsen KE. Multiplex PCR method for detection of *Clostridium difficile tcdA*, *tcdB*, *cdtA*, and *cdtB* and internal in-frame deletion of *tcdC*. J Clin Microbiol. 2011;49:4299–300.
6. Persson S, Torpdahl M, Olsen KE. New multiplex PCR method for the detection of *Clostridium difficile* toxin A (*tcdA*) and toxin B (*tcdB*) and the binary toxin (*cdtA*/*cdtB*) genes applied to a Danish strain collection. Clin Microbiol Infect. 2008;14:1057–64.
7. Grando D, Said MM, Mayall BC, Gurtler V. High resolution melt analysis to track infections due to ribotype 027 *Clostridium difficile*. J Microbiol Methods. 2012;89:87–94.
8. Lemee L, Dhalluin A, Pestel-Caron M, Lemeland JF, Pons JL. Multilocus sequence typing analysis of human and animal *Clostridium difficile* isolates of various toxigenic types. J Clin Microbiol. 2004;42:2609–17.
9. Balassiano IT, dos Santos-Filho J, Vital-Brazil JM, et al. Detection of cross-infection associated to a Brazilian PCR-ribotype of *Clostridium difficile* in a university hospital in Rio de Janeiro, Brazil. Antonie van Leeuwenhoek. 2011;99:249–55.
10. Stubbs SLJ, Brazier JS, O'Neill GL, Duerden BI. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J Clin Microbiol. 1999;37:461–3.