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Molecular epidemiology of Clostridioides (previously Clostridium) difficile isolates from a university hospital in Minas Gerais, Brazil

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ABSTRACT

The molecular epidemiology of 38 non-duplicate toxigenic Clostridioides (previously Clostridium) difficile isolates from inpatients from a hospital in Brazil during a 6-year period (2012-2017) were investigated by multilocus sequence typing (MLST) and ribotyping. These isolates were classified into 20 sequence types (ST), six (30%) of which were novel, revealing a high diversity in a single hospital. Classic hipervirulent strains ST1/RT027 and ST11/RT078 were not identified, while ST42 (almost all RT106) was the most common type, being detected in 11 (28.9%) strains. Noteworthy, six (15.8%) isolates were classified into five STs from clade 2, four of which were new ST and RT. Our study suggests that possible hypervirulent strains other than ST1/RT027 might be inadvertently circulating in Brazilian hospitals and highlights the importance of permanent surveillance on circulating strains in a national scale.

Keywords: Hipervirulent Clostridium difficile; Nosocomial diarrhea; MLST

NOTE

Clostridioides (previously Clostridium) difficile is known as the main infectious cause of antibiotic-associated diarrhea worldwide (Burke and Lamont, 2014). Since the early 2000s, the incidence and severity of C. difficile infection (CDI) have increased dramatically in some countries. This finding has been linked to the emergence of hypervirulent strains, such as those from PCR ribotype (RT) 027 and RT078, which are classified by multilocus sequence typing (MLST) in clades 2 and 5, respectively (Kuijper et al., 2006; Knight et al., 2015).

The epidemiology of CDI is highly dynamic as new strains continue to emerge worldwide (Munoz et al., 2017). Considering both the geographical differences of CDI epidemiology and the potential worldwide spread of epidemic isolates, epidemiological studies to characterize currently circulating strains and permanent surveillance are necessary (Janezic, Rupnik, 2015; Costa et al., 2016). Despite this, circulating strains in Latin America countries are largely unknown (Balassiano et al., 2012). Specifically in Brazil, several studies in the last decade failed to detect RT027 and RT078 in humans (Balassiano et al., 2009; Balassiano et al., 2011; Monteiro et al., 2014; Silva et al., 2015; Cançado et al., 2018). Although these known hypervirulent ribotypes seems not frequent in our country, studies have identified several binary toxin (CDT) positive strains in humans and animals in Brazil, some from new ribotypes (Silva et al., 2015; Costa et al., 2016; Cançado et al., 2018). This finding is of concern once the production of binary toxin is frequently associated with hipervirulent strains (Rodriguez et al., 2016) and with increased lethality and recurrence (Berry et al., 2016). Thus, more detailed molecular studies become necessary to clarify if there are similarities of these isolates with previously reported hypervirulent strains (Gerding et al., 2014). We therefore aimed to evaluate 38 non-duplicate toxigenic C. difficile isolates by MLST in order to better understand the epidemiology of CDI in a Brazilian hospital.

From January 2012 to December 2017, a total of 38 non-duplicate toxigenic C. difficile isolates was obtained from inpatients with CDI at the Clinical Hospital of the Federal University of Minas Gerais, a 500-bed quaternary care hospital of Belo Horizonte, Minas Gerais state, southeast Brazil. Samples were obtained from patients ≥18 years who have received systemic antibiotics anytime in the last 3 months and presented acute diarrhea

after 72 hours or more of hospitalization. During the time this study was conducted (2012-2017), the institution did not perform the diagnosis of CDI in the daily routine, thus these patients were selected by an active search for possible cases of CDI. All isolates were previously subjected to a multiplex-PCR for a housekeeping gene (tpi), the toxin A gene (tcdA), the toxin B gene (tcdB) and a binary toxin gene (cdtB) (Silva et al., 2011). For Multilocus Sequencing Typing (MLST), the amplification and sequencing reaction of the seven loci of MLST followed the procedures proposed by Griffiths et al. (2010). The analysis of sequences were made in program Unipro UGENE 1.28 (UniPro, Russia) and the MLST profiles were obtained in the public database https://pubmlst.org/cdifficile/. Isolates were PCR ribotyped as previously described (Janezic and Rupnik, 2010). Ribotypes not identified in the Brazilian C. difficile library were sent to the National Reference Laboratory for C. difficile (University of Leeds, United Kingdom) and a new type number was assigned.

MLST revealed 20 different STs, including six novel STs and five new allele sequences. Of these six novel STs, four were also identified as new ribotypes (Table 1). The diversity found in the present study seems to be higher than previously reported in other hospitals elsewhere (Weber et al., 2013; Kuwata et al., 2015; Cheng et al., 2015; Nicholas et al., 2017; Wang et al., 2017; Chen et al., 2018). Some studies have showed a link between the high diversity of ribotypes and less occurrence of CDI by RT027 and RT078 (Freeman et al., 2015; Rodriguez et al., 2016). Interestingly, classic hipervirulent strains (ST1/RT027 and ST11/RT078) were not identified in the present work. Anyway, taking together the high diversity and high percentage of new STs identified (6 out of 20 STs, 30%), the present work suggests a high diversity in our institution.

ST42, almost all classified as RT106, was the most common strain identified in the present study (Table 1), corroborating previous studies in other Brazilian hospitals (Balassiano et al., 2009; Balassiano et al., 2011; Secco et al., 2014; Silva et al., 2015; Costa et al., 2017; Cançado et al., 2018). ST42/RT106 is also endemic in Colombia (Salazar et al., 2018); over one decade ago this ribotype was endemic in United Kingdom hospitals (Vohra and Poxton, 2011; Wilcox et al., 2012; Davies et al., 2016). ST2, all classified as RT014/020, was the second most common strain. This finding is also in accordance with previous studies which indicates RT014/020 as a worldwide disseminated ribotype (Sundram et al., 2009; Weber et al., 2013; Yan et al., 2013; Cheng et al., 2015; Kuwata et al., 2015; Nicholas et al., 2017).

Although the classic hypervirulent strains ST1/RT027 and ST11/RT078 were not identified in the present work, six strains were classified into clade 2, the same clade as some hypervirulent strains (Figure 1). Despite one strain, identified as ST114/RT111, all other isolates from clade 2 were novel STs and novel ribotypes. In the present study, one strain was also identified as ST11/RT126, being the first report of a clade 5 strain in humans in Brazil. RT126 is genetically related to RT078 and was recently associated with severe symptoms in a patient with recurrent CDI (Sachsenheimer et al., 2018). Interestingly, patients infected with isolates classified into clade 2 or 5 or patients infected with cdtB+ strains were younger than those infected with strains from clade 1 (P=0.009) and cdtB- isolates (P=0.0128), respectively. It is well-known that patients aging >65 years old are in higher risk factor for CDI (Eze et al., 2017) and the risk of severe outcome and mortality are also known to increase with age (Miller et al., 2010). Some studies have also suggested that patients with community acquired CDI have a tendency to be younger those with health care associated CDI (Rao et al., 2015). Contrasting with the present

report, Aitken et al. (2016) found that patients infected with RT027 were younger than those infected with non-RT027. Despite that, there are few studies focusing on the association between C. difficile strains and patient age.

Similar to the present report, the majority of previous Brazilian studies failed to detect RT027 or RT078 in humans (Balassiano et al., 2009; Balassiano et al., 2011; Monteiro et al., 2014; Silva et al., 2015; Cançado et al., 2018). So far, RT027 was only recently reported in two patients from a hospital in the south Brazilian region, one of which was classified into ST67 (Pires et al., 2018). There is also a description of a clade 2 strain in a hospital in the northeast region of Brazil (Costa et al., 2016). Interestingly, this strain was classified as ST41 and also belonged to a new ribotype 821(CE). Taking together, these results reinforce the need for broader studies in Brazil to characterize currently circulating strains and the molecular and clinical epidemiology of CDI on a national scale.

Among the six strains classified into the clade 2, two isolates were classified as ST461/RT883. These isolates were obtained from inpatients that developed CDI within a 14-days interval from each other. Thus, to better understand the similarity of these two strains, they were submitted to a double-locus sequence typing (DLST), a technique that involves the sequencing of two highly variable loci (TR6 and C6) and it is known to be more discriminatory that MLST and ribotyping (Stojanov et al., 2016; Oliveira Junior et al., 2018). DLST was performed as previously described by Stojanov et al. (2016), and the sequences were analyzed using the BioNumerics software version 7.0 (Applied Maths, Belgium). Interestingly, both strains were classified into the same DLST (9_44), suggesting a close molecular relationship.

Recently, the emergence of the clade 2 strain ST244/ST41 was linked to outbreaks with high mortality rate in Australia, calling attention for the importance of constant vigilance, especially related to strains from clade 2 (Lim et al., 2014). Interesting, the analysis of the concatenated sequences of the clade 2 identified in the present study revealed these isolates are related to ST1 and ST41, but there are more genetic similarities among each clade-2 Brazilian isolate than to the classic hypervirulent strains (Figure 2). In addition, in some European countries, studies have shown that, after few years, ST2/RT014/020 and ST42/RT106 incidences have markedly decreased, with a clear shift towards ST1/RT027 predominance (Davies et al., 2016). This finding corroborates previous hypothesis that dominant C. difficile strains might be excluded by so-called hypervirulent ribotypes, including non-RT027 strains (Goorhuis et al., 2009; Yakob et al., 2015). It is also important to remember that in some Asia countries, where RT027 and 078 are rare, other ribotypes, mostly 017 and 018, emerged as responsible for outbreaks (Collins et al., 2013). Taking these works and the present study together, it is clear the need for an active vigilance on C. difficile epidemiology in Brazil. Also, as already advocated by other authors, the vigilance should not focus only RT027 and RT078 once emergence of other strains, including possible epidemic C. difficile isolates, seems constant (Smits et al., 2013).

The present study revealed a high diversity of C. difficile in the studied hospital, including new strain types and new ribotypes. In addition, while most previously studies on Brazil focused on the detection of RT027 or RT078 in humans, our study suggests that other possible hypervirulent (clade 2) strains are also circulating in this Brazilian hospital. Further studies are necessary to elucidate if this same epidemiology occurs in other Brazilian healthcare institutions. Thus, the present work highlights the importance of

permanent vigilance and reinforces the need for broader epidemiological studies in Brazil to characterize currently circulating strains on a national scale.

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