Prevalence and molecular types of *Clostridium difficile* isolates from faecal specimens of patients in a tertiary care centre

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*Clostridium difficile* infection (CDI) leads to considerable morbidity and mortality among hospitalized patients. Faecal specimens from 1110 hospitalized patients suspected for CDI were cultured for isolation of *C. difficile* and characterization of virulence genes. PCR was carried out for toxigenic genes *tcdA*, *tcdB*, *cdtA* and *cdtB* and PCR-RFLP for *fliC* and *slpA* genes. Of 174 (15.7 %) *C. difficile* isolates, 121 (69.5 %) were toxigenic, amongst which 68 (56.2 %) also had both *tcdA* and *tcdB* genes. The remaining 53 (43.8 %) of the isolates also had at least one of the toxin genes. Binary toxin genes (*cdtA* and *cdtB*) with only one of the two components were present in 16 (9.2 %) of the 174 isolates. The other virulence genes – *fliC* and *slpA* – were present in 100 % of the isolates. The most frequent PCR-RFLP type of *fliC* gene was type I (*n* = 101), followed by type VII (*n* = 49) and type III (*n* = 24). The *slpA* gene presented with three combinations of patterns. Characterization of virulence genes in *C. difficile* isolates is of extreme importance for epidemiological surveillance and control of outbreaks owing to the capacity of this bacterium to adapt to new environmental circumstances, leading to the emergence of new epidemic strains.

INTRODUCTION

*Clostridium difficile* is an important cause of nosocomial as well as community diarrhoea. *C. difficile* infection (CDI) is generally self-limiting but may progress to the potentially fatal pseudomembranous colitis, leading to considerable morbidity and mortality. The hospital environment and patients undergoing treatment with antibiotic or other drugs provide favourable conditions where spores of *C. difficile* persist and virulent clones thrive. There has been a marked increase in healthcare-acquired diarrhoea since the late 1990s to early 2000s, more so in elderly patients (McDonald et al., 2006; Burckhardt et al., 2008). The dramatic change in the epidemiology of CDI during recent years, in both frequency and severity, owing to the emergence of virulent strains like NAP1/BI/027 (North American Pulse Field type 1/Restriction Endonuclease Assay type BI/Ribotype 027) in North America (Cartman et al., 2010), ribotype 078 in Europe (Goorhuis et al., 2008) and ribotype 017 in Asia (Collins et al., 2013), has made *C. difficile* a public health concern.

When an individual is exposed to *C. difficile*, the organism adheres to the colonic mucosa with the help of flagella and tissue-binding surface layer proteins (SLPs), which could contribute to the host tissue degradation and to dissemination of CDI (Péchine et al., 2005). Flagella are composed of flagellin (*FliC*), which acts as a virulence factor and is present in both flagellated and non-flagellated *C. difficile* strains (Tasteyre et al., 2001). The *fliC* gene is therefore a good genetic epidemiological marker of CDI. SLP, encoded by the *slpA* gene, also constitutes a reliable target for differentiation of *C. difficile* isolates. Both these genes can be easily amplified from various strains by the PCR method, the amplified DNA digested with restriction enzymes and profiles compared.

Clinically significant strains of *C. difficile* produce one or both of toxin A (*TcdA*) and toxin B (*TcdB*), recognized as the main virulence factors of the pathogen. The genes encoding *TcdA* and *TcdB* are *tcdA* and *tcdB*, respectively. In addition to their contribution to disease, *TcdA* and *TcdB* are the primary markers for diagnosis of CDI and are
detected in the stools of patients by antigenic assays, cytotoxicity assays and genetic amplification techniques. Another toxin identified in \textit{C. difficile} is the binary toxin encoded by the two genes \textit{cdtA} and \textit{cdtB}, which act synergistically (Rupnik et al., 2003); this toxin has the potential to act in conjunction with TcdA and TcdB or to act alone in so-called ‘non-toxicogenic’ strains (Geric et al., 2003). Schwan et al. (2009) suggested that the binary toxin might increase the adherence and colonization of \textit{C. difficile}.

Molecular methods have been used to characterize \textit{C. difficile} strain prevalence, as the clinical significance of culture remains unclear owing to asymptomatic carriage of \textit{C. difficile} (Jain et al., 2013). From the limited studies on CDI available from Asia (Collins et al., 2013), \textit{C. difficile} has been observed as a low prevalence hospital pathogen in the continent (Burke & Lamont, 2014). There has been hardly any molecular work conducted to characterize the virulence genes of circulating \textit{C. difficile} strains prevalent in India, probably owing to lack of funding and difficulty in culturing the pathogen in the laboratory. The local prevalence and the significance of binary toxin in \textit{C. difficile} isolates also need to be investigated. In the present study, we investigated the prevalence and molecular types of \textit{C. difficile} strains isolated from faecal specimens of CDI patients from a tertiary care centre in North India.

### METHODS

This prospective study was conducted in the Department of Gastroenterology (Division of Microbiology), Postgraduate Institute of Medical Education and Research, a 2100 bed tertiary care teaching hospital at Chandigarh, India, to which patients from different parts of North India (Chandigarh, Punjab, Haryana, Himachal Pradesh, Jammu and Kashmir, western parts of Uttar Pradesh and some parts of Rajasthan) are referred. The study was approved by the institute’s ethics committee and was carried out from June 2012 to December 2014. Written informed consent was taken from all the patients or their wards.

**Patient population.** A total of 1110 patients >2 years of age, who developed diarrhoea after >72 h of admission to various wards of the hospital and were suspected of CDI, formed the basis of investigation. Demographic information and usage of antibiotics and other drugs by the patients during the past 2 weeks were recorded. The patients were evaluated for signs and symptoms of CDI and underlying diseases.

During analysis the patients were categorized into the following four groups according to their age: a paediatric group (n=189), young adult group (n=504), middle age group (n=342) and geriatric group (n=75), as published earlier (Singh et al., 2015).

**Culture for \textit{C. difficile}**. A single faecal specimen from each diarrhoeal patient suspected of CDI was cultured for \textit{C. difficile} anaerobically for 48 h at 37 °C after enrichment in Robertson’s cooked meat medium. \textit{C. difficile} isolates were identified phenotypically by routine Gram staining, UV fluorescence and biochemical reactions, as well as molecularly by identification of the triose-phosphate isomerase (\textit{tpi}) gene specific for \textit{C. difficile} (Singh et al., 2015).

**Molecular investigations.** For molecular investigation of \textit{C. difficile}-specific genes for adhesins and toxins, \textit{C. difficile} ATCC 43255 and NAP1/BI/027 strains were used as positive controls and \textit{Clostridium perfringens} MTCC 13124 served as a negative control. Primers used in PCR for \textit{C. difficile}-specific as well as toxin genes, and restriction enzymes used for PCR-RFLP of adhesins are given in Table 1. For PCR assay in general, the 20 μl PCR mixture contained 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl pH 8.3, 1.5 mM MgCl₂), 0.2 mM of 10 mM solutions of each of dATP, dCTP, dGTP and dTTP.

### Table 1. Primers and restriction enzymes used in this study

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
<th>Sequence 5′→3′</th>
<th>Amplicon size (bp)</th>
<th>Restriction enzymes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{tdcA}</td>
<td>\textit{Ta1F}</td>
<td>ATGATAAGGCAACCTTCAGTG</td>
<td>904</td>
<td>–</td>
<td>Scott et al. (2002)</td>
</tr>
<tr>
<td>\textit{tdcA}</td>
<td>\textit{Ta2R}</td>
<td>TAAGTTCCTCCTGGCTCATTCA</td>
<td>591</td>
<td>–</td>
<td>Wren et al. (1993)</td>
</tr>
<tr>
<td>\textit{cdtA}</td>
<td>\textit{tdcBF}</td>
<td>AAGGTGTATATGGATGAT</td>
<td>353</td>
<td>–</td>
<td>Stubbs et al. (2000)</td>
</tr>
<tr>
<td>\textit{cdtA}</td>
<td>\textit{tdcBR}</td>
<td>TAACACTTTATACACAATT</td>
<td>490</td>
<td>–</td>
<td>Stubbs et al. (2000)</td>
</tr>
<tr>
<td>\textit{cdtB}</td>
<td>\textit{cdtAF}</td>
<td>TGAACCTGGAAAAGGTGATG</td>
<td>342</td>
<td>–</td>
<td>Karjalainen et al. (2002)</td>
</tr>
<tr>
<td>\textit{cdtB}</td>
<td>\textit{cdtAR}</td>
<td>AGGATTATTTACTGGACCATTTT</td>
<td>342</td>
<td>–</td>
<td>Karjalainen et al. (2002)</td>
</tr>
<tr>
<td>\textit{flaC}</td>
<td>\textit{flaC F}</td>
<td>ATGAGAGTTTAAATGTTGAGTGC</td>
<td>870</td>
<td>HindIII, DraI, RsaI</td>
<td>Tasteyre et al. (2000)</td>
</tr>
<tr>
<td>\textit{flaC}</td>
<td>\textit{flaC R}</td>
<td>CTATCCTAAATTTGTAAGTGC</td>
<td>870</td>
<td>HindIII, DraI, RsaI</td>
<td>Tasteyre et al. (2000)</td>
</tr>
<tr>
<td>\textit{sfpA}</td>
<td>\textit{sfpA F}</td>
<td>ATGATAAGGCAACTTTCTAGTG</td>
<td>1500</td>
<td>PvuII</td>
<td>Karjalainen et al. (2002)</td>
</tr>
<tr>
<td>\textit{sfpA}</td>
<td>\textit{sfpA R}</td>
<td>TAAGTTCCTCCTGGCTCATTCA</td>
<td>1500</td>
<td>HindIII, DraI, RsaI</td>
<td>Karjalainen et al. (2002)</td>
</tr>
</tbody>
</table>
PCR, and typing of 94° was performed for 25 cycles of 94°. DNA was carried out at 95°.

Identification of virulence genes of C. difficile. Evaluation of C. difficile isolates for tcdA, tcdB, cdtA and cdtB genes was done by PCR, and typing of fliC and slpA genes was done by PCR-RFLP.

PCR for tcdA and tcdB genes of C. difficile. PCR amplification of tcdA and tcdB genes of C. difficile was carried out, with the first step at 94°C for 3 min to ensure denaturation of the sample. Amplification was performed for 25 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 15 s for toxin A. For toxin B the amplification steps were similar to those of toxin A, except that the last step at 72°C was carried out for 45 s.

PCR for binary toxin cdtA and cdtB genes. Binary toxin genes cdtA and cdtB were amplified by using primers and conditions as described by Stubbs et al. (2000). Reactions were subjected to 25 cycles of 94°C for 45 s, 52°C for 30 s and 72°C for 30 s, with a final extension step for 10 min.

Identification and typing of fliC and slpA genes. PCR-RFLP was used to study the variability of the fliC gene encoding flagellin, as described by Karjalainen et al. (2000). In brief, initial denaturation of DNA was carried out at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, followed by a final extension at 72°C for 10 min. Amplified products were digested with HindIII, DraI, RsaI and HinfI restriction enzymes.

The variable domain of the slpA gene encoding the SLP of C. difficile was amplified by PCR-RFLP as described by Karjalainen et al. (2002). Briefly the initial denaturation was carried out at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 45°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 10 min. Amplified products were digested with PvuII, HinfI, DraI and RsaI restriction enzymes.

RESULTS

Relationship of C. difficile toxigenicity with other parameters

C. difficile was isolated from 174 (15.7%) of a total of 1110 faecal specimens. All the microbiologically identified C. difficile isolates (100%) were also positive for the species-specific tpi gene. Of these, 121 (69.5%) isolates were toxigenic, i.e. they possessed either the tcdA (Fig. 1a) or the tcdB gene (Fig. 1b) or both. Usage of antibiotics was found to be significant (P=0.001) as a risk factor [odds ratio (OR)=1.811] for causing watery diarrhoea. But there was no correlation between toxigenic C. difficile positivity and watery diarrhoea.

Among the toxigenic isolates, 68 (56.2%) possessed both of the toxigenic genes (tcdA and tcdB) and the remaining 53

Fig. 1. PCR products of tcdA and tcdB genes of C. difficile, sizes 624 and 591 bp, respectively. (a) Gene tcdA: lane 1, 100 bp ladder; lane 2, positive control; lanes 3–7, C. difficile isolates; lane 8, negative control. (b) Gene tcdB: lane 1, 100 bp ladder; lane 2, negative control; lane 3, positive control; lanes 4–8, C. difficile isolates.
(43.8%) had one of the toxin genes. Only the toxin A ($tcdA^+ tcdB^-$) gene was found in 13 (10.7%) and only the toxin B ($tcdA^- tcdB^+$) gene in 40 (33.1%) of the toxigenic isolates. Toxicogenic C. difficile was more frequently present in males (72.0%) than in females (28.0%). Toxicogenic C. difficile was not found to be significant ($P=0.658$) in any age group by the Pearson chi-squared test. Though no correlation was found between the usage of antibiotics and toxigenic C. difficile positivity, toxigenic C. difficile was not found to be significant ($P<0.05$) more frequently isolated from patients on antibiotics than from those not on antibiotics. On comparing the number of patients with underlying diseases and the presence of toxigenic isolates, toxigenicity was highly associated with patients with cancers (77.3%), followed by patients who had undergone surgery (75.0%) and those who had gastrointestinal disorders (70.5%).

**Identification of binary toxin genes**

Binary toxin genes ($cdtA$ and $cdtB$) with only one of the two components (Fig. 2) were present in 16 (9.2%) of the 174 isolates. The $cdtA$ gene was present in nine (5.2%) and the $cdtB$ gene in seven (4.0%) of these isolates. On checking the relationship of the binary toxin genes with $tcdA$ and $tcdB$, it was observed that two (12.5%) and five (31.3%) isolates were positive for $cdtA$ and $cdtB$ genes, respectively. One (6.3%) isolate was positive for only $cdtA$ and $tcdA$ genes, whereas five (31.3%) were positive for $cdtA$ and $tcdB$ genes. One (6.3%) isolate was positive only for $cdtB$ and $tcdB$ genes. One (6.3%) isolate each was positive for $cdtA$ and $cdtB$ genes and negative for both $tcdA$ and $tcdB$ gene components.

**Identification of fliC gene**

The amplification of the fliC gene gave a single product of an 870 bp fragment in 100% of C. difficile isolates (Fig. 3). After digestion with HindIII, DraI and HinfI restriction enzymes, three different restriction profiles were obtained, whereas four restriction profiles were obtained with RsaI endonuclease (Fig. 4). The most frequent RFLP type found among the isolates was type I ($n=101$), followed by type VII ($n=49$) and type III ($n=24$).

**Identification of slpA gene**

The slpA gene was found in 100% of the 174 isolates, with an amplicon size of approximately 1500 bp (Fig. 5). All the isolates revealed RFLP profiles of the slpA gene which were identified as three combinations of patterns and labelled as A ($n=119$), B ($n=37$) and C ($n=18$).

**Ribotypes.** The ribotypes of the 121 toxigenic isolates were 001, 017 and 106, while those of the 53 non-

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**Fig. 2.** PCR amplification of $cdtA$ and $cdtB$ genes of C. difficile, sizes 490 and 353 bp respectively. Lane 1, 100 bp ladder; lanes 3 and 5, positive control; lanes 2, 4, 6 and 7, samples.

**Fig. 3.** PCR detection of fliC gene (870 bp) of C. difficile. Lane 1, 100 bp marker; lane 2, negative control; lane 3, positive control; lanes 4–8, C. difficile isolates.
toxigenic isolates were 009 and 010, in which slpA types A and B were more frequent. Table 2 shows the correlation of slpA and fliC RFLP types with ribotypes and binary toxin in toxigenic and non-toxigenic C. difficile.

**DISCUSSION**

A trend of increasing prevalence and severity of CDI has been reported in Europe and the USA during the past 10 years due to the acquisition of hypothetical functions associated with laterally acquired DNA (Quesada-Gómez et al., 2015). No outbreak of CDI has been noted in this region (Northern India) or in any other part of India. In an earlier study comprising 3044 patients with suspected CDI, we investigated both hospitalized patients and out-patients, among which 17.5% were positive for faecal C. difficile toxins by ELISA (Vaishnavi et al., 2015). The focus of the present study was mainly to investigate the virulence genes in C. difficile isolated from hospitalized patients who developed diarrhoea >72 h after admission.

C. difficile can be molecularly identified on the basis of the housekeeping tpi gene, and all our C. difficile culture isolates possessed the tpi gene (Singh et al., 2015). Other workers have also reported the presence of the tpi gene in C. difficile isolates (Lemee et al., 2004) or faecal samples (Nawar et al., 2014), confirming the accuracy and reliability of PCR species identification of C. difficile (Lemee et al., 2004; Nawar et al., 2014). The prevalence of CDI was reported to be higher in Qatar than in Europe; though comparable to other Middle Eastern countries (Al-Thani et al., 2014). Comparatively, in Europe C. difficile infections are responsible for only 3.6% of all hospital-acquired infections (ECDC, 2015). In our study, C. difficile positivity was 13.8% in the paediatric group, 17.0% in the young adult group, 15.0% in the middle age group and 14.7% in the geriatric group, as published earlier (Singh et al., 2015). Antibiotic resistance was largely observed towards clindamycin (57.5%), and ciprofloxacin (38.5%), but was significantly low towards metronidazole (1.72%) and nil (0%) towards vancomycin (Singh et al., 2015).

The diagnosis of CDI along with clinical opinion requires the detection of toxigenic C. difficile. Molecular methods

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**Fig. 4.** (a, b) PCR-RFLP pattern of fliC gene digested with HindIII, DraI, Rsal and Hinfl.

**Fig. 5.** PCR detection of slpA gene (~1500 bp) of C. difficile. Lane 1, 100 bp marker; lane 2, negative control; lane 3, positive control; lanes 4 and 5, C. difficile isolates.
targeting toxigenic genes have come into prominence for
detection of toxin-producing isolates. In the present
study, 69.5% of C. difficile isolates carried the toxin
genes. Toxigenic C. difficile positivity was not found to be
significant in any of the groups, suggesting that all age
groups are equally at risk. This is probably due to exposure
to the hospital environment and decreased resistance to
pathogens. Toxigenic C. difficile was isolated most fre-
quently from patients with cancers, followed by patients
who underwent surgery and those who had gastrointestinal
disorders, confirming that these patients are at great risk of
acquiring toxigenic pathogens.

There is substantially increased reporting of the presence
of the variant toxin strain A⁻B⁺ among clinical isolates
(Barbut et al., 2002; Drudy et al., 2007). A low incidence of
C. difficile A⁻B⁺ strains (0.2%) was reported from the
USA (Lyerly et al., 1992), 6.2% from Europe (Barbut et al.,
2007) and 2.3% from Canada (Martin et al., 2008). How-
ever, a higher incidence of the variant A⁻B⁺ strains was
reported from different parts of Asia. In Shanghai, 33.3% of
the isolates were A⁻B⁺ strains, whereas in Stockholm
there was no A⁻B⁺ strain (Huang et al., 2009). Kim et al.
(2010) reported the A⁻B⁺ variant in 25.7% of C. difficile
isolates in Korea. Goudarzi et al. (2013) reported the
prevalence of A⁻B⁺, A⁺B⁻ and A⁺B⁺ strains as 85.3, 6.7
and 8%, respectively, in Iran. In the present study, there
was a successful discrimination between isolates possessing
tcdA⁻tcdB⁺, tcdA⁺tcdB⁻ and tcdA⁻tcdB⁺. The tcdA gene
was present alone in 10.7% of C. difficile isolates whereas
33.1% were positive for the tcdB gene alone, which
correlated with other Asian studies. Among the toxigenic
isolates, those with only the toxin B (tcdA⁻tcdB⁺) gene
were more in number than those with only the toxin A
(tcdA⁺tcdB⁻) gene, suggesting that variant strains of C.
difficile with toxin B alone may also cause the disease,
though variants with toxin A are also important. Thus the
presence of variant toxin strains highlights the need to use
C. difficile diagnostic methods that can detect both toxins
individually.

Binary toxin genes are present in NAP1/BI/027 owing to
gene exchange among several clostridial species (Voth
& Ballard, 2005). Up to 2% of C. difficile produce only
binary toxin (Geric et al., 2003) and 4–12% of C. difficile
isolates are positive for the toxin (Geric et al., 2003;
Stubbs et al., 2000). The prevalence of binary toxin genes
in both toxigenic and non-toxigenic C. difficile strains has
been reported from various countries, such as the USA
(0.2%) (Geric et al., 2003), Spain (4.5%) (Alonso et al.,
2005) and Poland (8.6%) (Pituch et al., 2005). During a
hospital outbreak of CDI in Pittsburgh, USA, up to 65%
of the clinically recovered C. difficile isolates had binary
toxin genes (McEllistrem et al., 2005). Doosti & Mokhtari-
farsi (2014) reported that only 1.1% of the C. difficile
isolated from calves contained all four toxin
genes, namely tcdA, tcdB, cdtA and cdtB. Recently, Eckert
et al. (2015) documented that C. difficile isolates producing
only binary toxin were pathogenic despite the lack of TcdA
and TcdB. In the present study too, toxigenic C. difficile
isolates with variant toxin genes were present. Also, the
prevalence of binary toxin genes (9.2%) was similar to
that reported in other studies. Interestingly, in the present
study, the isolates harboured only one of the two compo-
ents (cdtA and cdtB) of the gene, implying thereby
that binary-toxin-positive isolates carrying both the genes
are absent in this geographical location, as in other Asian
countries (Collins et al., 2013). As intact binary toxin
genes are a characteristic of NAP1/BI/027, the hyperviru-
 lent strain of C. difficile is obviously absent in this region.
The role of binary toxin as a virulence factor is still unclear
and could have implications for laboratory diagnostics,
which rarely include testing for binary toxin.

Flagellated bacterial strains have better capacity for adhe-
rence to intestinal epithelial cells and subsequent coloniza-
tion of the gut than strains without flagella. Tasteyre
et al. (2000) found nine different groups of fliC corre-
sponding to numerous serogroups. In the present study,
the fliC gene was found in 100% of the C. difficile isolates
and three different restriction profiles were obtained. Type
I isolates, considered to be either toxin-positive or toxin-
negative, were found in a higher number of isolates than
type VII isolates, which are mostly toxin-positive (Tasteyre
et al., 2000). This correlated well with the low prevalence
of toxin-positive isolates in the region. Similarly, C. difficile
SLP with binding properties may be involved in immune
recognition of the pathogen (Ryan et al., 2011).
study from Japan, Kato et al. (2005) reported that C. difficile culture-negative faecal samples were positive for the slpA gene of this species. Thus, direct typing of the slpA gene can be useful when there are a small number of C. difficile present in the clinical materials, and can also be helpful in global epidemiological sharing of typing data from clinical isolates identified from different laboratories. Ribotypes of C. difficile identified in this region were the same as those found in other parts of Asia.

The detection of toxins by PCR assays is gaining popularity in diagnostic testing because of the increased sensitivity and specificity available by this method (Vaishnavi, 2010). Even though the cost of PCR is relatively high, the results are more reliable and more accurate than traditional methods (Humphries, 2012). The fliC gene is a good marker for epidemiological and phylogenetic studies, and the variable region of the slpA gene could be employed to differentiate between the serogroups of C. difficile (Karjalainen et al., 2002) and for determining its immunogenic properties for vaccine development. Further analysis is necessary to decipher the differences in the role of variant fliC and slpA genes amongst the C. difficile strains. C. difficile has the capacity to adapt to new environmental circumstances, which leads to the emergence of new epidemic strains. Molecular typing of the isolates will be useful during epidemiological investigations of C. difficile outbreaks and sporadic cases of the disease.

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