Phenotypic Characterization of Non-toxigenic \textit{Clostridioides difficile} Strains Isolated From Patients in Mexico

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\textit{Clostridioides difficile} is a Gram positive, sporulated, rod-shape, anaerobic pathogen responsible for nosocomial diarrhea and colitis, mainly in antibiotic treated patients. \textit{C. difficile} produce two toxins responsible for disease, toxin A (TcdA) and toxin B (TcdB), although not all strains produce them. Non-toxigenic \textit{C. difficile} (NTCD) strains are able to colonize the intestinal mucosa and are often isolated from asymptomatic individuals. NTCD are poorly studied, their evolutionary history has not been elucidated, and their relationship with illness remains controversial. The aim of this work was to analyze the phenotype of NTCD strains isolated from clinical cases in hospitals of México, and whether NTCD strains present characteristics that differentiate them from the toxigenic strains. Seventy-four \textit{C. difficile} strains isolated from patients were tested for cytotoxicity and 14 were identified as NTCD strains. We analyzed phenotypical characteristics that are important for the biology of \textit{C. difficile} like colony morphology, antibiotic resistance, motility, sporulation, and adherence. Strains were also genotyped to determine the presence of genes coding for TcdA, TcdB and binary toxin and ribotyped for 027 type. When compared with toxigenic strains, NTCD strains presented an enlarged branched colony morphology, higher resistance to metronidazole, and increased sporulation efficiency. This phenotype has been reported associated with mutations that regulates phenotypic characteristics like swimming, sporulation or adhesion. Our results show that phenotype of NTCD strains is heterogeneous but still present characteristics that differentiate them from toxigenic strains.

Keywords: phenotype, \textit{Clostridioides difficile}, non-toxigenic, hospital-acquired diarrhea, Mexico

INTRODUCTION

\textit{Clostridioides difficile} has emerged as a healthcare problem worldwide, \textit{C. difficile} colitis has been one of the most costly and common causes of diarrhea during the last 20 years, causing millions of deaths every year (Garey et al., 2010). The infection may present different clinical characteristics, including diarrhea, pseudomembranous colitis, fulminating colitis, toxic megacolon, and even death...
(Burke and Lamont, 2014). An important factor to consider in the transmission of *C. difficile* infection (CDI) is the asymptomatic carriage of strains, which has been reported in up to 18% of asymptomatic people (Donskey et al., 2015). To understand the mechanism by which this pathogen causes disease, it is necessary to know the ecology of the microorganism. *C. difficile* was considered as part of the normal intestinal microbiota, until 1970 when it was identified as an opportunistic pathogen. The administration of antibiotics disrupts the composition of gut microbiota causing a dysbiosis which results in reduced resistance to pathogens and *C. difficile* spores takes advantage of this to germinate and colonize the host.

Clostridioides difficile has several virulence factors, including toxins A and B encoded on a pathogenic island called PaLoc (Pathogenesis Locus) (Dingle et al., 2014). A third toxin, denominated CDT, which belongs to a family of toxins called binary toxins was recently identified in toxigenic *C. difficile* strains (Perelle et al., 1997; Gulk et al., 2001). The disruption of cytoskeleton, and cellular damage caused by the action of toxins A and B leads to the dissociation of thigh junctions between colonocytes, promoting the loss of epithelial integrity and consequently diarrhea (Hunt and Ballard, 2013). However, there are some strains that are unable to produce one or the two toxins and are denominated as Non-toxigenic *C. difficile* (NTCD). These NTCD are classified into three groups: the first does not contain the PaLoc genes, the second present a modified PaLoc and are unable to produce toxin A or B; finally, the third group produces very low amount of toxins and their cytotoxic activity is not detected (Fluit et al., 1991; Wren et al., 1993; Cohen et al., 1998; Brouwer et al., 2012; Natarajan et al., 2013). NTCD are usually isolated from asymptomatic individuals, but there are some reports on the association of NTCD strains with diarrhea (Martirosian et al., 2004). In addition, NTCD strains have been isolated from patients also infected with toxigenic strains (Miyajima et al., 2011; Behroozi et al., 2013) suggesting that NTCD may be involved in mixed infections. Other reports highlight the ability of NTCD to protect against toxigenic *C. difficile* strains, although the mechanisms providing this protective effect are unknown (Nagar et al., 2013). Of note, recent works suggest that the use of NTCD strains as an oral probiotic would be an effective treatment for *C. difficile* associated diarrhea, an option probably simpler than fecal transplant (Villano et al., 2012; Nagar et al., 2013; Gerdig et al., 2015; Arruda et al., 2016). Thus, the role of NTCD strains in gut disease remains unclear and should be better studied. The aim of this study was to gain more information about phenotypic and genotypic characteristic of NTCD strains isolated from patients with hospital-acquired diarrhea.

**MATERIALS AND METHODS**

**Isolation and Identification of *C. difficile* From Patients**

All samples were obtained from patients suffering from hospital-acquired diarrhea, attended at the National Medical Center “XXI Century” of the Mexican Institute of Social Security, Mexico City. Stools were cultured on CCFA agar after an ethanol shock and incubated under anaerobic conditions (N2, 5% CO2, and 5% H2) for 48–72 h at 37°C. Isolates were purified by passages of single colonies on Casman Blood Agar (Difco; Becton, Dickinson and Company, United States) and CCFA agar at least three times. *C. difficile* strains were identified by Gram staining, morphological growth in blood agar, UV-fluorescence and PCR amplification of 16S rRNA and tpi genes. Isolates were also subcultured in meat broth (Difco; Becton, Dickinson and Company, United States) for storage.

The amplification of 16S rRNA gene was performed using primers PS13 and PS14 (**Table 1**), while tpi gene amplification was performed with tpi-Fw and tpi-Rv primers in a 25 µl reaction according to the GoTaq Green Master Mix Protocol (Promega, United States). Thermocycler conditions were 7 min at 95°C, followed by 30 cycles of 30 s at 94°C, 90 s at 57°C and 1 min at 72°C; then a final extension of 7 min at 72°C. Products were analyzed by 2% agarose electrophoresis stained with Eva Green 1X (Jena Bioscience, Germany).

**DNA Extraction**

Bacteria were cultured in BHI broth (Bacto; Becton, Dickinson and Company, United States) under anaerobically atmosphere for 24 h at 37°C. Then cultures were centrifuged and pellets were used for extraction of bacterial DNA using InstaGene Matrix (BioRad, Mexico) following manufacturer’s recommendations. DNA concentration and quality were measured using a Nanodrop spectrophotometer, ensuring that A260/280 values were greater than 1.8.

**Detection of Virulence Genes by PCR and Ribotyping**

PCR amplification of the tcdA, tcdB, and cdtB genes was performed in 25 µl reaction with GoTaq Green Master Mix (Promega, United States), using the primers described in **Table 1**. Thermocycler conditions were 15 min at 95°C, followed by 30 cycles of 30 s at 94°C, 90 s at 57°C and 60 s at 72°C, with a final extension of 7 min at 72°C. We also designed primers to amplify the complete tcdC gene (**Table 1**) using the following conditions, 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 42.5°C and 1 min at 72°C, with a final extension of 4 min at 72°C. Products were analyzed by 2.0% agarose electrophoresis, stained with Eva Green 1X (Jena Bioscience, Germany). Additional genotyping of the strains was done by ribotyping as described previously (Bidet et al., 1999). DNA from a clinical *C. difficile* strain ribotype 027 (Kindly donated by Dra. Elvira Garza, Monterrey, Mexico) (Camacho-Ortiz et al., 2015) was included in each run as a reference. Amplification products were subjected to electrophoresis in a 2.5% agarose gel, ethidium bromide stained and analyzed under ultraviolet light using the LabWorks Image Acquisition and Analysis Software (Version 4.5.00.0 for Windows, UVP).
**In vitro Cytotoxicity Assays**

VERO (ATCC CCL-81) and HT-29 (ATCC HTB-38) cells were grown at a confluence of 70% in DMEM medium (In Vitro, Mexico) supplemented with 5% fetal calf serum (GIBCO, United States). The sensitivity of each cell line to the *C. difficile* toxins is different, VERO cells are more susceptible for toxin B, whereas HT-29 cells are more susceptible to toxin A (Torres et al., 1992). Briefly, *C. difficile* strains were grown 48 h in BHI media under anaerobic conditions. Then 50 µl of filtered supernatants were added onto monolayer of cells grown in 96-well plates under a 5% CO₂ atmosphere. Cytotoxicity was recorded under the microscope after 24 and 48 h of incubation at 37°C. To confirm specificity of the cytotoxic effect, a neutralization assay with anti-toxin was done, the cytotoxic supernatants were incubated 1 h with the anti-toxin antibodies (Remel, United States) in a 1:1 ratio, before added to Vero cells. Neutralization of cytotoxicity was recorded after 24 h of incubation at 37°C under a 5% atmosphere.

**Colony Morphology**

This assay was made as previously reported (Mackin et al., 2013) with slight modifications. Briefly, strains were grown overnight in *Brucella* broth at 37°C under anaerobic atmosphere. Then, 3 µl of culture were spotted onto BHI agar plates (Bacto; Becton, Dickinson and Company, United States) and incubated for 7 days to allow growth. Morphology of the single colonies was analyzed macroscopically. We used seven clinical toxigenic strains as controls (MX080, MX081, MX091, MX111, MX164, MX295, and MX300) as well as the ATCC 9689 and the 027/NAP1 *C. difficile* reference strains.

**Sporulation Assays**

A conventional spore recovery assay was used to evaluate the sporulation rate of the strains as previously reported, with some modifications (Garneau et al., 2014). *C. difficile* strains were grown on Brucella broth for 48 h anaerobically at 37°C, bacterial concentration was adjusted to 0.5 McFarland turbidity and 3 µl were inoculated in previously reduced soft BHI agar (0.15% agar). Samples were then alcohol shock by mixing 1.0 ml of bacterial suspension with 80% ethanol and incubated for 45 min. Samples were then diluted in Brucella broth and 10-fold serial dilutions were plated onto Cassman Blood Agar. Plates were incubated for 48 h anaerobically and then CFU were measured. Sporulation efficiency was determined using the following formula:

\[
\text{Sporulation efficiency(\%)} = \frac{\text{Spores}}{\text{Vegetative cells} + \text{spores}} \times 100
\]

**Adherence Assay**

The ability of adherence of *C. difficile* strains were assayed on Vero cells. Vero cells were cultivated in culture bottles containing M199 medium (GIBCO, United States) supplemented with 5% fetal calf serum (GIBCO, United States), and grown until a confluence of 70%. Then cells were passed to a 96-well plates and cultured until a confluence of 90%. The cells were washed three times with PBS (Merk, Germany), and

| Name        | Reference                | Sequence (5′–3′)                  |
|-------------|--------------------------|-----------------------------------|
| cdtB-F1     | Mulvey et al., 2010      | TGG ACA GGA AGA ATA ATT CTT TC    |
| cdtB-F2     | Mulvey et al., 2010      | TGC AAC TAA CGG ATC TCT TGC      |
| tcdA-F      | Lemee et al., 2004       | AGA TTC CTA TAT TTA CAT TGC AAT AT |
| tcdA-RA3B   | Lemee et al., 2004       | AAC ATC AAT CTC GAA AAG TCC AC   |
| tcdB-F3     | Murray et al., 2009      | AAT GCA TTT TTT ATA AAC ACA TGG  |
| tcdB-F4     | Murray et al., 2009      | AAG TTT CTA ACA TCA TTT CCA      |
| tcdCEcoFw   | This work                | TAA ATA TCT AAT AAA AGG GAG AAT CTA TTA TCT CTG CTA AAA AAA ATG GTA AOG |
| tcdCBamRv   | This work                | GCT GTA GAG AAA ATT TAC TAT GGA GGA AAT TAC TAT GGA TCA TTA TTA TAT ATT GTA GTC |
| PS13        | Persson et al., 2008     | GGA GCG AGC AGT GGG GAA TA       |
| PS14        | Persson et al., 2008     | TGA GGG GCG GTG TGT ACA AG       |
| tpi-Fw      | Lemee et al., 2004       | AAA GAA GCT AGT AAG GGT ACA AA   |
| tpi-Rv      | Lemee et al., 2004       | CAT AAT TAA GGG TCT ATT CCT AC    |
infected with *C. difficile* strains (10^7 bacteria/mL). Infected cells were incubated for 1 h under anaerobic conditions at 37°C, and washed four times with PBS to removed non-adherent bacteria. Attached bacteria were then removed with 100 µL of 0.06% Triton X-100 for 30 min at 37°C, and 1:10, 1:100, and 1:1000 dilutions were plated onto Cassman blood agar, and incubated anaerobically at 37°C for 48 h. Adherence efficiency was calculated using the following formula:

\[
\text{Adherence efficiency(\%)} = \frac{\text{Adherent bacterial cells}}{\text{Initial inoculum} + \text{adherent bacterial cells}} \times 100
\]

**Statistical Analysis**

The statistical significance (p-value) for all experiments was calculated with one-way ANOVA test. A Tukey t-test was used to analyze the different NTCD genotypes studied. A p < 0.05 was considered to be significant. Statistics were done with the ASTATSA calculators.

**RESULTS**

**Isolation of NTCD Strains**

A total of 74 *C. difficile* strains isolated from patients were tested for cytotoxicity and 14 of these strains were identified as NTCD strains, since they had no cytotoxic effect on either, Vero (Supplementary Figure S1) or HT29 cells (data not shown). Thus, in 19% of the clinical cases with hospital-acquired diarrhea we identified only infection with NTCD strains; of note, in these patients no mixed infection with toxigenic strains was found and no other enteropathogen was identified. Among these 14 strains 7 were isolated from adults and 7 from children. To corroborate the identity of these strains, all isolates were confirmed as *C. difficile* by PCR for the 16S rRNA and *tpi* gene. Then the presence of toxin genes was analyzed by PCR, and we found the two previously reported genotypes (Natarajan et al., 2013): 9 strains with all three toxin genes absent (*cdtB−tcdA−tcdB−*) and 5 strains with all three genes present (*cdtB+tcdA+tcdB+*). These results indicate that the lack of cytotoxicity is not always due to the absence of the genes. To further characterize these strains we also determined the presence of the tcdC gene in all strains and found that it was present in all 5 *cdtB+tcdA+tcdB* strains, but absent in all 9 *cdtB−tcdA−tcdB−* strains (data not shown). Ribotyping of the 14 isolates showed that 4 of the 5 *cdtB+tcdA+tcdB+* strains were 027 type, whereas only one of the 9 *cdtB−tcdA−tcdB−* strains was 027 (Supplementary Figure S2). Clinical and genotype characteristics of the strains are present in Table 2.

**Colony Morphology**

Once we selected NTCD strains we compared their colony morphology after growing them for 5 days in BHI agar plates. Our results showed that colonies of NTCD were usually larger than colonies of toxigenic strains, and presented more ruffled edges (Figure 1), with some strains displaying highly branched colonies like strains MX113 and MX249. The size of NTCD colonies had an average of 1.89 cm, while toxigenic colonies had an average of 0.65 cm, a difference that was statistically significant (p < 0.05). Among the NTCD strains no difference in size was observed between those *cdtB+cdtA+tcdB+* and those *cdtB−cdtA−tcdB−*.

**Antibiotic Susceptibility**

Non-toxigenic *C. difficile* strains were tested for sensitivity to clindamycin, metronidazole, levofloxacin and vancomycin using the threshold suggested by EUCAST and CLSI: > 16 µg ml⁻¹ for Clindamycin, > 2.0 µg ml⁻¹ for metronidazole, > 2.0 µg ml⁻¹ for vancomycin, and > 4.0 µg ml⁻¹ for levofloxacin. NTCD strains with the *cdtB+tcdA+tcdB* genotype showed a significantly increase resistance to metronidazole (p < 0.05) (Figure 2) when compared with *cdtB−cdtA−tcdB−* and with the cytotoxigenic strains. Cytotoxigenic strains presented higher resistance to levofloxacin and clindamycin than the NTCD strains, although difference did not reach statistical significance.

**Motility**

In order to learn more about the phenotypic characteristic of NTCD strains, we analyzed the motility of the strains. The extent of the growth was measured after 18 h in BHI soft agar. Our results showed no difference in motility between the genotype and the capacity of swimming (Figure 3), although we found some strains that had an increased swimming ability (strains MX113, MX024, and MX041 and the 027 control strain), whereas others presented a very reduced motility (strains MX153 and MX249).

**Spore Formation in NTCD Strains**

Sporulation is one of the most important advantages that *C. difficile* has as a pathogen. The spores provide the ability to

| No. (%) | Strains |
|---------|---------|
| Sex     |         |
| Female  | 7 (50.00) | MX019, MX025, MX066, MX107, MX115, MX126, MX154 |
| Male    | 7 (50.00) | MX041, MX151, MX153, MX184, MX249, MX367, MX501 |
| Age     |         |
| Adult   | 7 (50.00) | MX019, MX025, MX066, MX107, MX125, MX153, MX154 |
| Infant  | 7 (50.00) | MX041, MX113, MX151, MX184, MX249, MX367, MX501 |
| Genotype |         |
| cdtB+tcdA+tcdB+/tcdC+ | 5 (35.71) | MX019, MX041, MX066, MX151, MX154 |
| cdtB−tcdA−tcdB−/tcdC− | 9 (64.29) | MX025, MX107, MX113, MX125, MX153, MX184, MX249, MX367, MX501 |
resist disinfectants, antibiotics, hostile environment and are the vehicle for *C. difficile* transmission. Previous studies reported that sporulation levels of non-toxigenic strains were lower than the levels displayed by toxigenic strains (ref). In contrast, we found that whereas NTCD *tcdA*−*cdtB*−*tcdB*− strains showed a high capacity of spore formation, NTCD *tcdA*+*cdtB*+*tcdB*+ strains had a deficient spore formation capacity, and the toxigenic strains displayed a moderate capacity (*p* < 0.05) (Figure 4).

**Adherence of NTCD Strains in Vero Cells**

We analyzed the adherence ability of NTCD to Vero cells (Figure 5). The assay was done under anaerobic conditions as
FIGURE 3 | Non-toxigenic C. difficile motility. Range motility of C. difficile non-toxigenic and toxigenic strains. Cells were grown in soft BHI agar (0.15% agar) for 18 h, and swimming range was measured. Gray bars: cdtB+ tcdA+ tcdB+ strains. White bars: cdtB- tcdA- tcdB- strains. Striped bars: control toxigenic strains. Values represent the average ± standard deviation (SD) from three independent experiments. *p > 0.05.

previously described. Results showed that most strains presented a high adherence efficiency, and only two had a reduced ability, below 20% (MX125 and MX295). There was no significant difference between genotypes and adherence.

DISCUSSION

Non-toxigenic C. difficile strains are poorly studied, and there is a need to learn more about the physiology and evolution of this type of C. difficile strains and to understand their role in disease. To our knowledge, this work is the first report on phenotypic characteristics of NTCD strains aiming to differentiate toxigenic from NTCD C. difficile strains. All the strains included in this work are clinical isolates from patients that presented hospital-acquired diarrhea and where no other enteropathogen was identified. In addition, in these cases we did not find mixed infection with toxigenic C. difficile strains, suggesting the possibility that NTCD strains may be associated with some cases of hospital-acquired diarrhea. This suggestion needs to be further studied by thoroughly characterizing the enterotoxicity of NTCD strains in cell and animal models.

In spite of the phenotypic heterogeneity of C. difficile strains, we were still able to identify some phenotypic characteristics that differentiate NTCD from toxigenic strains, in particular in their colony morphology, sporulation, and antimicrobial resistance. When we analyzed the colony morphology of NTCD strains, we identified that NTCD strains have more ruffled edges. Previous studies reported that this colony phenotype resulted after mutation in genes like the transcription–repair coupling factor, mfd (Willing et al., 2015b), the master regulator spo0A (Mackin et al., 2013), the transcriptional regulator recV, or the wall protein cwpV (Reynolds et al., 2011). Although it is known that Mdf protein is a repair factor, studies in C. difficile have shown that it may also have a role in transcriptional regulation, suppressing CodY and CcpA effect. In toxigenic strains, the mutation of mdf gene increase toxin production, and induce a change in colony morphology, although the factor responsible for this change in morphology is not known (Willing et al., 2015a). On the other hand, Spo0A protein has an important role in the control of sporulation (Underwood et al., 2009) and of toxin production in several strains (Mackin et al., 2013). A recent work reported that mutation of spo0A has an effect on colony morphology and motility across a solid surface, like the twitching motility mediated by type IV pili (Mackin et al., 2013). Thus, it is possible that NTCD strains may present mutations in genes that result in altered colony morphology but also in altered toxin production and in sporulation efficacy. In this sense, it is noteworthy that NTCD strains with the genotype tcdA− cdtB− tcdB− presented the highest efficacy in sporulation, although a previous study found no correlation between the genotype and spore variability (Burns et al., 2010). Sporulation may be regulated by as much as 314 genes (Fimlaid et al., 2013), and one of them is the spo0A gene. Our results in colony morphology and spore efficacy suggest that spo0A gene may be mutated in NTCD strains and that this mutation could also be responsible for the nontoxic activity of these strains. The study of the genomes of NTCD strains will help clarify the genetic bases of these phenotypic characteristics; in this sense, we are in the process of sequencing the genomes of the NTCD strains reported in this work.

We observed that NTCD tcdA+ cdtB+ tcdB+ strains were more resistant to metronidazole than the tcdA− cdtB− tcdB− and
FIGURE 4 | Sporulation efficiency of non-toxigenic Clostridioides difficile strains. Sporulation efficiency by chemical shock with 80% ethanol. Gray bars: cdtB⁺tcdA⁺tcdB⁺ strains. White bars: cdtB⁻tcdA⁻tcdB⁻ strains. Striped bars: control toxigenic strains. Values represent the average ± standard deviation (SD) from three independent experiments. *p < 0.05.

FIGURE 5 | Adherence of NTCD strains in Vero cells. Monolayers of Vero cells were incubated with each strain. Cell adherence efficiency was measured as described previously. Gray bars: cdtB⁺tcdA⁺tcdB⁺ strains. White bars: cdtB⁻tcdA⁻tcdB⁻ strains. Striped bars: control toxigenic strains. The data presents and standard deviation are for three independent experiments. p > 0.05.
the toxigenic strains. The resistance of C. difficile toxigenic strains to metronidazole has been previously reported, and it has been suggested that this may have a high impact on the pathophysiology of recurrent CDI diseases (Richardson et al., 2015). There is a report on a single NTCD strain with a stable resistance to metronidazole where resistance was associated with an altered pfo gene expression. Pfo is a pyruvate-flavodoxin oxidoreductase that participate in the response to oxidative stress (Moura et al., 2014). Since in our study most metronidazole resistant strains were NTCD tcdA−cdtB+tdcB+, it will be interesting to see if there is any association between resistance to metronidazole and inhibition of toxin expression.

The ability to adhere to cells and the motility were phenotypes that were not different among the strains that we studied, regardless of cytotoxicity or genotype. Previous studies have shown that some toxigenic strains that do not have flagella, they still conserve late flagellar genes that are also responsible for regulation of toxin production (36). It will be of interest to study the role of flagellar genes in our NTCD strains to better understand the processes of cytotoxicity, motility, adherence, and colonization in C. difficile.

The natural history of the infection by C. difficile NTCD strains remains unclear, and their participation in the pathogenesis of intestinal disease is yet unknown. In this study we characterized NTCD strains isolated from patients with hospital-acquired diarrhea, which suggest that they could cause disease even in the absence of toxin production. We identified two NTCD genotypes, one PCR positive for all three toxins (tcdA+cdtB+tdcB+) and the other PCR negative for the toxins (tcdA−cdtB−tdcB−). We do not know if tcdA+cdtB+tdcB+ strains have functional genes and are able to produce toxin in vivo in the human intestine. Of note, we detected the presence of the tcdC negative regulator gene in the 5 tcdA+cdtB+tdcB+ NTCD strains, which suggest that probably these strains have a deficient positive regulator cdtR gene. On the other hand, the strains tcdA−cdtB−tdcB−, may be able to produce unknown virulence factors, which elucidation requires further studies. Also, we found that most of the tcdA+cdtB+tdcB+ NTCD strains were of the ribotype 027, the ribotype most commonly reported for toxigenic strains. Our results suggest 027 is common in strains with the toxin genes, even if the show negative for cytotoxicity. On the other hand, most of the tcdA−cdtB−tdcB− were not 027, which is also in agreement with previous reports (Urban et al., 2001; Pereira et al., 2016; Riley et al., 2018). Only one isolate (strain 153) was negative for toxins by PCR but still ribotype 027, a result that is not agreement with previous reports (Valiente et al., 2014); and we need to further study this isolate. A work in progress is the sequencing of the genome of these NTCD isolates, which will help clarify if this strain has truncated or mutated toxin genes that make it PCR negative.

CONCLUSION

In spite of the diversity in the phenotypic characteristics among the studied strains, there were significant differences in colony morphology, metronidazole resistance, and sporulation between NTCD and cytotoxigenic strains. Colony morphology and sporulation differentiated between NTCD and cytotoxigenic strains, whereas metronidazole resistance differentiated NTCD tcdA+cdtB+tdcB− strains from NTCD tcdA−cdtB+tdcB− and from toxigenic strains. We are currently sequencing the genome of both toxigenic and NTCD strains to better understand the genomic characteristic of both types of strains.

ETHICS STATEMENT

This project was evaluated and authorized by the Bioethics Committee of the “Hospital de Pediatría, Instituto Mexicano del Seguro Social” with the registration number R2015-785-089. All participants provided written informed consent, and in the case of children under 16 years, their parents were responsible for it.

AUTHOR CONTRIBUTIONS

MR-C and JT designed the experiments. MR-C and MS-R carried out all experiments. MR-C, MC, and JT wrote the manuscript and analyzed the data. All authors have critically read, corrected, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00084/full#supplementary-material

FIGURE S1 | Cytotoxic analysis of C. difficile strains in Vero cells. After cytotoxic analysis, cells were washed with PBS and fixed with methanol. Then Giemsa staining was performed to analyze the cytotoxic effect in Vero cells.

FIGURE S2 | Ribotyping of NTCD strains. PCR ribotype analysis from the non-toxigenic strains from this study.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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