A MLST Clade 2 Clostridium difficile strain with a variant TcdB induces high levels of histologic inflammatory damage, epithelial disruption and oxidative response

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Running head: New Clostridium difficile ribotype with variant TcdB

ABSTRACT

The epidemiology of Clostridium difficile infections is highly dynamic as new strains continue to emerge worldwide. Here we present a detailed analysis of a new C. difficile strain (ICC-45) recovered from a cancer patient in Brazil that died from severe diarrhea. Through WGS, ribotyping, PFGE, and toxinotyping, we determined that strain ICC-45 is toxigenic ($tcdA^+$, $tcdB^+$, and $ctdB^+$) and belongs to ST41 from MLST Clade 2 and toxinotype IX. In addition it does not belong to any know PCR-ribotype and PFGE-type, and toxinotype IX. Strain ICC-45 encodes for a variant TcdB that induces a distinct CPE in agreement with its toxinotype. Unlike epidemic NAP1/027 strains, which are also classified to MLST Clade 2, strain ICC-45 is susceptible to fluoroquinolones and does not overproduce toxins TcdA and TcdB. However, supernatants from strain ICC-45 and a NAP1/027 strain produced similar levels of pro-inflammatory cytokines, epithelial damage, and oxidative stress response in the mouse ileal loop model. These results indicate that inflammation and oxidative stress seem to be common features in the pathogenesis of Clade 2 strains. Finally, this work contributes to the description of differences in virulence among various C. difficile strains.

Keywords: Clostridium difficile, MLST Clade 2, variant toxin B, inflammation, oxidative stress response

1. Introduction

Clostridium difficile is a strict anaerobic, spore-forming, toxin-producing Gram-positive bacterium. This bacterium is the main cause of nosocomial diarrhea associated with antibiotic use [1]. Most strains of this pathogen produce an enterotoxin denominated toxin A (TcdA) and toxin B (TcdB) which have a cytotoxic activity. In addition, a small subset of strains also synthesize an actin-ADP–ribosylating toxin known as binary toxin (CDT) [2]. Genes encoding TcdA and TcdB are located on the chromosome along with tcdR, tcdE, and tcdC in a phage-derived pathogenicity locus of 19.6 kB (PaLoc). The genes for the CDT toxin, by contrast, are located elsewhere on the chromosome [3]. Since 2003, many countries have reported outbreaks of C. difficile infections (CDI) [4–6]. This high incidence of CDI at a global scale has been linked to the emergence and rapid spread of epidemic strains, such as those from the PFGE-type NAP1, ribotype 027, and multilocus sequence type 01 (NAP1/027/ST01) [7,8]. Among the factors that likely contribute to the increased virulence of NAP1/027 strains are their i) capacity to produce the three aforementioned toxins, ii) resistance to fluoroquinolones, and iii) toxin overproduction [9]. Furthermore, it has also been shown that their TcdB induces a classical, arborizing cytopathic effect on different cell lines [10].

According to MLST and comparative genomics, there are five major clades of C. difficile. Under this scheme all hypervirulent strains, including NAP1/027 strains, are classified into MLST Clade 2 [8,11]. However, the epidemiology of this pathogen is changing [12] and new epidemic or more virulent lineages are starting to emerge in different parts of the world [13–15].

To expand current knowledge on the etiology and pathogenesis of CDI, we present here a comprehensive analysis of the molecular epidemiology of a C. difficile strain from a MSLT Clade 2, new PFGE-type and ribotype that was isolated from a cancer patient

that died from severe diarrhea. Pathogenesis studies of this strain and a NAP1/027 strain were assessed in the mouse ileal loop model.

2. Materials and methods

2.1 C. difficile isolation

C. difficile isolation was attempted on stool samples positive for C. difficile toxins (Xpect C. difficile toxin A/B test. Oxoid, Hampshire, United Kingdom) collected between May 2013 and November 2013 in a reference Cancer Hospital (Ceará Cancer Institute, Fortaleza, Brazil). Samples were treated with 96% ethanol and inoculated onto Cefoxitin-Cycloserine-Fructose agar plates (Oxoid) that were later incubated for 5 days in jars under anaerobic conditions (90% N_2, 10% CO_2). Colonies were identified phenotypically by RapID 32A system (bioMérieux, Lyon, France) and by PCR-amplification of the tpi gene [16].

2.2 PCR-based genotyping

Genomic DNA from each strain was obtained from overnight cultures in Brain Heart Infusion broth (BHI; Oxoid) using the InstaGene reagent (Bio-Rad, Hercules, CA). Fragments of tcdA, tcdB, cdtB, and tcdC were amplified by PCR using known primers and conditions [16].

2.3 PFGE typing

The PFGE procedure used was derived from published protocols [17]. Images were analyzed with the BioNumerics software (version 5.1. Applied Maths, Austin, TX) and the resulting macrorestriction patterns were compared to those deposited in the databases of the National Microbiology Laboratory of the Public Health Agency of Canada (Dr. Michael Mulvey. Winnipeg, Canada).

2.4 Ribotyping, whole genome-based analyses, and MLST

For ribotyping, intergenic spacer regions were amplified using Bidet primers as described previously [18]. PCR-ribotypes were determined by submitting data to the web-database WEBRIBO (http://webribo.ages.at) and using the database of Maribor (Dr. Rupnik). The whole genome sequence (WGS) of strain ICC-45 was obtained using multiplexed paired-end libraries (2 x 300 bp) and sequencing-by-synthesis on MiSeq (Illumina, San Diego, CA). Reads were assembled using Velvet [19]. The resulting scaffolds were ordered using MAUVE [20] and the genome of the NAP1/027 reference strain R20291 (FN545816). For automatic annotation, we used Prokka [21] and custom C. difficile databases. For core genome multialignment, variant calling, and core genome phylogeny we used the Harvest suite [22]. PaLoc and TcdB sequences were extracted manually and aligned with MAFFT [23] or MUSCLE [24]. For these subsequences, phylogenetic reconstruction through maximum-likelihood was done using Fasttree [25]. For MLST typing, we used the MLST 1.7 tool maintained by the Center for Genomic Epidemiology at the Danish Technical University [26] and the classification scheme based on the genes adk, atpA, tpi, glyA, dxr, sodA, and recA proposed by Griffiths et al. [11]. The sequences reported in this article have been deposited in EMBL/GenBank under the accession numbers SAMN04011654 (ICC-45), SAMN04011636 (LIBA-5809), SAMN04011647 (LIBA-6656), SAMN04011650 (LIBA-5757), SAMN04011652 (LIBA-5758) of Bioproject PRJNA293889.

2.5 Toxinotyping

For toxinotyping, A1 and B3 regions of tcdA and tcdB were analyzed with a method described previously [27].

2.6 Antibiotic susceptibility testing

Minimum inhibitory concentrations (MIC) for ciprofloxacin, ceftriaxone, moxifloxacin, levofloxacin, clindamycin, metronidazole, rifampin, and vancomycin were determined

using E-test (bioMérieux) [28]. Resistance breakpoints were set in agreement with the guidelines of the Clinical and Laboratory Standards Institute (M11-A8) as follows: ciprofloxacin > 4 µg/ml; ceftriaxone > 64 µg/ml; moxifloxacin > 4 µg/ml; levofloxacin > 4 µg/ml; clindamycin > 4 µg/ml; metronidazole > 16 µg/ml; vancomycin > 8 µg/ml; rifampin > 32 µg/ml. Mutations in gyrA and gyrB were identified manually through comparison of our WGS with that of the reference strains C. difficile 630 (fluoroquinolone^{S}) and R20291 (fluoroquinolone^{R}).

2.7 Preparation of bacterial cell-free supernatants

ICC-45 was grown in BHI for the indicated times. Thereafter, cells were harvested by centrifugation at 20.000 x g for 30 min and the resulting supernatant was passed through 0.2-µm pore-size filters [16]. For comparative purposes, the NAP1/027 strain (LIBA-5756) [29] was included in the experimental design because it belongs to MLST Clade 2. The NAP4/014-020 strain (ICC-01 from this study), by contrast, belongs to MLST Clade 1 and was recovered from the same hospital as ICC-45 during the study period. Strain ATCC 700057 is non-toxigenic.

2.8 Cytotoxicity assays on cell cultures

Decimal dilutions of bacterial cell-free supernatants were added to monolayers of HeLa cells grown in DMEM supplemented with 5% fetal bovine serum (Sigma). Cells were monitored for the appearance of cytopathic effects (CPE) by optical microscopy [30]. As a control, a specific TcdB antiserum (TechLab, Blacksburg, VA) was used to neutralize the effect of TcdB. Cytotoxicity was expressed as the inverse of the dilution of the supernatants that caused 50% cell rounding (CPE_{50}). Confluent HeLa cells grown on 12-mm glass slides were intoxicated with supernatants, immobilized, fixed, and the CPE induced was evaluated by phase contrast microscopy according to previously described protocols [31].

2.9 Toxin quantitation

The amount of toxins synthesized by the strains was quantified through Western Blotting of bacterial cell-free supernatants concentrated by methanol/chloroform precipitation [32]. To this end, proteins were separated in 7.5% SDS-PAGE gels and electro transferred to PDVF membranes which were later probed with monoclonal anti-TcdA (TTC8) or anti-TcdB (2CV) antibodies (tgcBIOMICS, Bingen am Rhein, Germany) [32]. Chemiluminescent signals emitted after addition of a goat anti-mouse IgG-horseradish peroxidase conjugate (Invitrogen, Carlsbad, CA) and the Lumi-Light Plus Western Blotting substrate (Roche) were recorded with a Chemidoc XRS documentation system (Bio-Rad).

2.10 Murine ileal loop model

The pathogenesis of the strains was compared using murine ligated ileal loops inoculated with bacterial cell-free supernatants. To this end, male Swiss mice of 20-25 g were fasted overnight and anesthetized with 60 mg/Kg ketamine and 5 mg/kg xylazine (König, Mairinque, SP, Brazil). Through a midline laparotomy, a 4-cm ileal loop was ligated and injected with 0.3 ml of supernatants or control solutions (TYT-broth or PBS). Mice were euthanized 4 h after inoculation and the length and weight of the intestinal loops were recorded [33]. These experiments were carried out according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and with permission of the Institutional Animal Care and Use Committee of the Faculty of Medicine of the Federal University of Ceará.

2.11 Histopathologic assessment of tissue sections

Intestinal sections were fixed in formalin and stained with hematoxylin-eosin for histopathological evaluation. Samples were evaluated for the severity of epithelial

damage, edema, neutrophil infiltration, and hemorrhage, using a scale ranging from 0 (absence of alterations) to 3 (severe) [34].

2.12 Detection of TNF-α, IL-1β and iNOS by immunohistochemistry

Immunohistochemistry-based assays for tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and the inducible isoform of nitric oxide synthase (iNOS) in ileal loop tissue sections were done using a previously described streptavidine-biotin-peroxidase method [35–37]. In this regard, TNF-α and IL-1β are pro-inflammatory cytokines and iNOS serves as an indicator of free radical production. Briefly, dewaxed tissue sections were treated with 0.05 M Tris-buffered saline (TBS) containing 3% normal horse serum and 0.3% Triton X-100 (Sigma). Then, they were rinsed with TBS buffer following incubation with rat anti-TNF-α (Sigma), rabbit anti-IL-1β (Santa Cruz Biotechnology, Dallas, TX) and rabbit anti-iNOS (Santa Cruz Biotechnology) primary antibodies. Colorimetric signals emitted after addition of a streptavidin-HRP-conjugated secondary antibody (Biocare Medical, Concord, CA) and the TrekAvidin-HRP Label + Kit (Biocare Medical) were recorded by optical microscopy.

2.13 Myeloperoxidase (MPO) assay

The extent of neutrophil accumulation in ileal tissue was estimated by measuring MPO activity as previously described [38]. Briefly, 100 mg of ileal tissue was homogenized in hexadecyltrimethylammonium bromide (HTAB) buffer. Then, the homogenate was centrifuged and MPO activity in the resuspended pellet was assayed by measuring the change in absorbance at 450 nm using O-dianisidine dihydrocloride and 1% H_2O_2 (Sigma). The results were reported as MPO units/mg of tissue. A unit of MPO activity was defined as that converting 1 mol of hydrogen peroxide to water in 1 min at 22°C.

2.14 Malondialdehyde (MDA) determination

Lipid peroxidation of intestinal cell membranes in the ileal loop was estimated by measuring the concentration of tissue MDA [39]. Briefly, MDA production was measured by means of a thiobarbituric acid reaction with intestinal tissue. Homogenized tissue was added to a thiobarbituric acid solution (Sigma), and the mixture was heated in a water bath at 100°C for 45 min. Thereafter, n-butanol (Sigma) was added and centrifuged at 40,800 x g for 15 min. The absorbance of the butanol layer was measured at 520 nm (Ab_1) and 535 nm (Ab_2) using a spectrophotometer. The concentration of MDA was calculated as $Ab_2 - Ab_1$ and expressed as nmol of MDA/g of intestinal tissue.

2.15 Glutathione (GSH) levels

GSH levels in the ileal loop were measured as a marker for antioxidant activity, according to the method described by Sedlak and Lindsay [40]. In these experiments, reduced-GSH concentrations indicate oxidative stress caused by inoculated supernatants. Ileal tissues were homogenized in an EDTA and trichloroacetic acid solution (Sigma). Samples were then centrifuged at 3,000 x g for 15 min at 4°C. Afterwards, Tris-buffer (pH 8.9) and 5,5-dithio-bis-2-nitrobenzoic acid solutions (Sigma) were mixed with the supernatants. Samples absorbance were measured at 420 nm and the results were reported as µg of GSH/mg of tissue.

2.16 Nitric oxide (NO) production

Nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction [41]. In brief, 200 mg of each intestinal sample were homogenized in a cold solution of potassium chloride (Sigma) and centrifuged at 40,800 x g for 15 min. Then, each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine

dihydrochloride in water); absorbance of the mixture was determined at 540 nm and the results were reported as a nitrite concentration (µM) according to a standard curve.

3. Results

3.1 Strain ICC-45 belongs to MLST Clade 2 but represents a new PFGE-type and ribotype

During the period under study four strains from two different PFGE macrorestriction pattern were isolated. Whereas 3 of those isolates were NAP4/RT014-20, the strain ICC-45 gave rise to a SmaI pattern without NAP designation (Fig. 1A). This unclassified strain was isolated from a 34-years old female patient diagnosed with breast cancer and metastasis to the nervous system. This patient was subjected to radiotherapy and had completed a chemotherapy cycle with Herceptin (Trastuzumab). Diarrhea began 16 days after hospitalization and she was treated with meropenem and vancomycin, evolving, however, to death after 54 days of hospitalization.

Further analyses revealed that strain ICC-45 corresponds to a new ribotype (Fig. 1B) and belongs to ST41 from MLST Clade 2. Congruently, a phylogenetic reconstruction based on core SNP confirmed that strain ICC-45 is closely related to epidemic NAP1/027/ST01 strains -such as the reference strain R20291 and LIBA-5756, a clinical isolate from a Costa Rican hospital [29]- as well as to clinical isolates from sequence types included in this Clade (ST67, ST154 and ST252) (Fig. 1C).

The tcdA restriction patterns obtained for ICC-45 and a NAP1/027 strain were identical. In contrast, we noted polymorphisms in the B1 fragment of tcdB, which includes the catalytic region of TcdB, among strains ICC-45 and NAP1/027. Hence the toxinotype of ICC-45 (IX) differed from that obtained for NAP1/027 strains (III).

Similar to NAP1/027 strains, strain ICC-45 possesses tcdA, tcdB and ctdB. However, it does not present the 18-bp deletion and the deletion at position 117 in tcdC characteristic of the former group of strains.

3.2 ICC-45 causes a variant cytopathic effect (CPE)

Cytotoxicity assays on HeLa cell, which primarily measure TcdB activity, revealed that ICC-45 supernatants produced the C. sordellii–like CPE caused by A-B+ strains [44]. This CPE was characterized by cell clumping, rounding, and cellular detachment (Fig 2). By contrast, the CPE induced by a NAP1/027 strain was a classic arborizing effect.

3.3 TcdB from strain ICC-45 resembles TcdB sequences of A-B+ strains

As anticipated from the previous results, the PaLoc sequences of ICC-45 and NAP1/027 strains were not identical. In general, TcdB from strain ICC45 exhibited higher identity to TcdB sequences from strains that induce variant CPEs, such as TcdB_8864 (95%) and TcdB_ST67 (100%), than to cognates from NAP1/027 strains (93%). Moreover, it showed the insertion at position 311 in TcdB that characterizes variant strains but not NAP1/027 strains. In line with this observation, the TcdB substrate recognition regions of strain ICC-45 and NAP1/027 strains only showed 62% of identity and the enzymatic domain of ICC-45 closely resembled those of variant TcdB strains (TcdB_8864 and TcdB_ST67). On the other hand, the toxin-binding and autoproteolitic domains of TcdB from ICC-45 and NAP1/027 strains showed 95-99% of identity (Fig. 3).

3.4 Strain ICC-45 lacks fluoroquinolone-resistance mutations

The MIC to fluoroquinolones of strain ICC-45 were lower than those recorded for epidemic NAP1/027 strains [42]. The susceptibility of strain ICC-45 to these antibiotics agrees with the absence of the Thr82Ile transition in GyrA that distinguishes the latter group of strains [43]. No mutations in gyrB were detected. Additionally, strain ICC-45 was resistant to ceftriaxone (>32 µg/mL) and clindamycin (8 µg/mL), but susceptible to

metronidazole (0.032 µg/mL), vancomycin (1 µg/mL), rifampicin (<0.002 µg/mL), moxifloxacin (2 µg/mL) and levofloxacin (2 µg/mL).

3.5 ICC-45 strain does not overproduce toxins

Compared to NAP4/014-020 strain, both ICC-45 and the NAP1/027 strains consistently presented higher cytotoxic titers. Nevertheless, the $CPE_{50\%}$ recorded for strain ICC-45 was lower than that obtained for the NAP1/027 strain tested (Fig. 4A). After 24 h of in vitro growth, the amount of TcdA in ICC-45 and NAP4/014-20 supernatants was rather low. In contrast, TcdB levels in strain ICC-45 were higher than in a NAP4/014-20 strain. High concentrations of both toxins were detected in the corresponding NAP1/027 supernatants (Fig. 4B).

3.6 ICC-45 and NAP1/027 strains induce equal levels of epithelial damage, inflammation, and oxidative stress response in the intestinal loop model

The pathogenesis of strain ICC-45 was assessed using the mouse ligated ileal loop model inoculated with bacterial-free supernatants. ICC-45 and NAP1/027 supernatants induced stronger secretion/edema measured by the normalized weight of the ligated ileal loop (Fig. 5 and Supplementary Fig. 1).

In addition, supernatants from ICC-45 and NAP1/027 strains induced higher histological damage scores than NAP4/014-020 and non-toxigenic control strains (Fig. 6). Moreover, ICC-45 and NAP1/027 supernatants elicited a strong inflammatory reaction (Fig. 6) and comparable levels of epithelial destruction, edema, neutrophil infiltration and hemorrhage (Fig. 7).

ICC-45 and NAP1/027 supernatants caused a significant increase in MPO activity in contrast to the NAP4/014-20 supernatant, which elicited a reaction similar to that from a non-toxigenic control (Fig. 8A). Congruently, TNF-α and IL-1β expression were

highly induced in ileal tissue exposed to ICC-45 and NAP1/027 supernatants, in contrast to other supernatants (Supplementary Fig. 2 and Fig. 3).

MDA tissue levels, determining lipid peroxidation process at the level of intestinal loop cells, were highly increased in ileal tissue exposed to ICC-45 and NAP1/027 supernatants (Fig. 8B). In addition, supernatants from ICC-45 and NAP1/027 strains induced a greater decrease in GSH levels and higher levels of nitrite than NAP4/014-20 and non-toxigenic strains, indicating an increased ability to induce oxidative stress by ICC-45 and NAP1/027 (Fig. 8C and 8D). Consistently, histological preparations showed higher expression of the inducible isoform of the nitric oxide synthase (iNOS) in intestines inoculated with ICC-45 and NAP1/027 supernatants (Supplementary Fig. 4). iNOS activity was detected mainly in epithelial cells of the ileum, although some lamina propia cells also presented this activity.

4. Discussion

Strain ICC-45 represents a new ribotype and PFGE-type. This isolate is phylogenetically related to NAP1/027/ST01 strains and other strains belonging to the MLST Clade 2 of the hypervirulent lineage postulated by Griffiths et al. [11]. Interestingly, strains from this clade have been reported in Australia (strain DLL3110 from RT244/ST41) [44] and recently in Latin America, as confirmed by this study and by the finding of other strains from sequence types 41, 67, 154 and 252 in Costa Rican hospitals (unpublished data). The fatal outcome of the patient, along with the responses elicited by strain ICC-45 in the murine ileal loop model, suggest that this strain has an increased pathogenic potential.

Although ICC-45 lacks some characteristics that have been associated to the increased virulence of epidemic NAP1/027 strain, like deletion in tcdC, toxin overproduction, and fluoroquinolone resistance, it induces inflammation and oxidative stress in levels

comparable to those induced by supernatants from the a NAP1/027 strain. A possible explanation for the increased pathogenic potential of strain ICC-45 in the intestinal loop model is the production of a variant TcdB. Indeed, variant TcdA-negative/TcdB-positive isolates have caused epidemics across several Asian countries [4,5]. Two important facts reinforce this notion: (i) toxin B has recently been proposed to be the main virulence factor in CDI and a primary factor for the induction of host innate immune and inflammatory responses [45] and (ii) other isolates from MLST Clade 2 synthetizing variant TcdB, such as the Australian strain RT244/ST41 [44], have been associated with severe disease.

It is tempting to speculate that even if ICC-45 produces less TcdB than NAP1/027 strains, variations in its substrate recognition domain contributes to the effects induced by the supernatant of this strain. It has been reported that variant TcdB modifies GTPases that are not glucosylated by other TcdBs [31]. Even more, the impact of distinct substrate modifications on the pathogenesis of C. difficile is only recently being explored, since it has been observed a broader pattern of GTPases glucosylation by TcdB from MLST Clade 2 strains, such as NAP1/027/ST01 and NAP1/019/ST67 [29], it could has a significant role in inflammatory response and epithelial damage.

The toxin-binding and autoproteolitic domains of TcdB from strain ICC-45 are similar to those of NAP1/027 strains. Differences in TcdB sequences from NAP1/027 strains have been associated with more efficient uptake and autoprocessing activities, and hence to an elevated cytotoxic potency [10]. These functional aspects could also be true for TcdB from strain ICC-45, and somewhat account for our results in cell cultures and the ileal loop model.

Our findings suggest that oxidative stress response, high production of iNOS, and secretion of pro inflammatory cytokines are important hallmarks of the increased

pathogenic potential displayed by strains from MLST Clade 2 such as ICC-45 and this NAP1/027 strain. Recent transcriptomic evidence from mice challenged with NAP1/027 strains reports that they induce Duox2 activation [45]. The Duox2 enzyme has homology with NADPH-oxidases and regulate oxidative responses [46]. In addition, ROS-mediated cell death has been considered an important feature of TcdB that contributes to the CDI pathogenesis [47].

IL-1β is one of the main cytokines involved in the immune response to C. difficile toxins [48] and its secretion has been related to the activation of the inflammasome via iNOS production [49]. Both ICC-45 and NAP1/027 strains induced a high secretion levels of this cytokine. Therefore, the increased virulent behavior of MLST Clade 2 strains could be attributed to the production levels and/or variation in TcdB as stated before; alternatively, other molecular factor(s) present in the supernatant and shared between strains belonging to MLST Clade 2 could have a role in the increased damage induce in the ligated-ileal loop model.

Another factor shared between ICC-45 and NAP1/027 strains is the production of CDT. Although the role of CDT is still not fully understood, recently it has been described that TcdB-negative strains harboring CDT induce CDI in an animal infection model, unlike strains that only harbor TcdA [45]. It is highly unlikely that TcdA plays a role in the increased pathogenic potential seen in ICC-45 and NAP1/027 strains since the former isolate produces low amounts of this toxins.

In conclusion, supernatants from ICC-45 and the NAP1/027 strains induced similar levels of pro-inflammatory cytokines, epithelial damage, and oxidative stress response in the mouse ileal loop model. These results indicate that inflammation and oxidative stress seem to be common features in the pathogenesis of MLST Clade 2 strains. The induction of oxidative response by the ICC-45 strain described in this work helps to

understand the differences in virulence seen among various C. difficile clinical isolates and the increased virulence phenotype attributed to the MLST Clade 2 lineage.

Competing interests

The authors declare that they have no competing interests.

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FIGURE LEGENDS

Fig. 1. Molecular typing and core genome-based analysis. (A) PFGE patterns from *C. difficile* strains isolated in a Brazilian Cancer Hospital. Two different *SmaI* macrorestriction patterns were detected and classified in the indicated NAP type. A previously undescribed NAP type is showed (ICC-45 strain). (B) Ribotyping of the strains indicated above. (C) A phylogenomic tree based on core SNPs revealed that strain ICC-45 is closely related to NAP1/027 strains and clinical isolates from the hypervirulent Clade 2. The genomes of reference strains R20291 (NAP1/027), M68 (NAP9/017 with a truncated tcdA and a variant toxin B) and NAP7/078 were included to validate the results of the typing methods.

Fig. 2. Morphological effect caused by bacterial cell-free supernatants from strain ICC-45 on HeLa cells. (A) Cells were initially treated with 10-fold dilutions of supernatants from 24 h cultures. Images obtained by phase contrast microscopy show cell rounding as well as cell detachment (variant effect) caused only by the ICC-45 strain. (B) TYT-broth was used as negative control.

Fig. 3. Alignment of TcdB amino acid residues sequences: i) variant TcdBs from reference strains 1470 and 8864, and clinical isolates from sequences types ST41 and ST67, ii) TcdB from a NAP1/027/ST01 clinical isolate and an epidemic NAP1/027 strain (R20291), and iii) TcdB of ST252 from MLST Clade 2. Black blocks represent disagreements to the TcdB sequence of strain R20291, which was selected as a reference for the alignment. The blue box depicts the glucosyltransferase region and the green box shows the autoproteolitic and the repetitive receptor binding domains, respectively.

Fig. 4. Cytotoxicity assays. (A) Quantification of TcdB production by the strains analyzed. Ten-fold dilutions of the supernatants were added to HeLa cells monolayers grown in DMEM supplemented with 5% fetal bovine serum. Cells were monitored for appearance of cytopathic effect (CPE) by optical microscopy. A specific TcdB antiserum was used to neutralize the effect of the toxin B. Cytotoxicity was expressed as the inverse of the dilution of the supernatants that caused 50% cell rounding in the monolayers (CPE_{50}). Bars represents the mean +/- s.e.m. of the CPE_{50} recorded for each strain. * $P < 0.05$ (One-way Kruskall-Wallis followed by Mann-Whitney U´s tests). (B) Amount of toxins secreted by the strains. Proteins from bacterial cell-free supernatants obtained after 24 h of growth in TYT-broth were concentrated and quantified by Western Blotting.

Fig. 5. Effect of bacterial cell-free supernatants of strain ICC-45 and other *C. difficile* genotypes on the weight/length of ligated mouse ileal loops. Bacteria-cell free supernatants (48 h growth) were prepared in TYT-broth. 6-8 ligated-ileal loops per group were inoculated. Four hours after inoculation mice were euthanized and the weight and length of their ileal loops were assessed. Data are presented as the ratio weight/length for each group. * $P < 0.05$ compared to BHI broth (One-way ANOVA with Tukeys´s correction).

Fig. 6. Histopathologic analysis of the effects elicited by bacterial-cell free supernatants of strain ICC-45 and other *C. difficile* genotypes on ligated mouse ileal loops. Samples from ligated ileal loops were stained with hematoxylin-eosin for histopathological evaluation (200X). (A) Strain ICC-45 caused edema of lamina propria and submucosa, inflammatory cell infiltration, focal mucosal disruption, and lumen hemorrhage. (B) A NAP1/027 strain showed alterations comparable to ICC-45. (C). A NAP4/014-20 strain

showed only discrete edema and inflammatory infiltration, (D) non-toxigenic strain and (E) PBS control.

Fig. 7. Quantification of histopathologic effects of bacterial cell-free supernatants of strain ICC-45 and other *C. difficile* genotypes on ligated mouse ileal loops. The severity of the histopathological alterations was scored in coded slides on a scale that ranged from 1 (mild) to 3 (severe). (A) Neutrophil infiltration, (B) edema, (C) epithelial damage, and (D) hemorrhage. * $P < 0.05$, compared to the groups without asterisk (Kruskall and Dunn's multiple comparison test).

Fig. 8. Effects of bacterial-cell free supernatants of strain ICC-45 and other *C. difficile* genotypes on MPO activity, MDA, GSH and nitrite levels measure in mouse ileal tissue. Samples from ligated ileal loops prepared as indicated in Figure 4 were analyzed for (A) myeloperoxidase MPO activity, (B) lipoperoxidation (MDA, tissular malondialdehyde), (C) GSH (glutathione), and (D) nitric oxide production. *$P < 0.05$, compared to the groups without asterisk (One-way ANOVA with Tukey´s correction).

Supplementary Fig. 1. Macroscopic appearance of mice ileal loops inoculated with supernatants from strain ICC/45 and other *C. difficile* genotypes. Ileal loops received 300 μL of supernatants from (A) strain ICC-45 strain, (B) NAP1/027, (C) NAP4/014-20 strains. (D) Non-toxigenic strain and (E) PBS. More blood was seen inside the loops in panels (A), (B) and (C) than in panels (D) and (E).

Supplementary Fig. 2. TNF-α expression in the mice ileal loops inoculated with supernatants from strain ICC/45 and other *C. difficile* genotypes. The pictures shown represent the immunohistochemistry of tissue sections from mice injected with supernatants of (A) strain ICC-45, (B) NAP1/027, (C) NAP4/014-20, and (D) a non-

toxigenic strains. As negative controls we tested PBS (E) or omitted addition of the primary antibody (F). Panels (A) and (B) show a greater number of labeled cells in the villi lining, crypts, and lamina propria. 400X magnification.

Supplementary Fig. 3. IL-1β expression in mice ileal loops inoculated with supernatants from strain ICC/45 and other *C. difficile* genotypes. The pictures shown represent the immunohistochemistry of tissue sections from mice injected with supernatants of (A) strain ICC-45, (B) NAP1/027, (C) NAP4/014-20, and (D) a non-toxigenic strains. As negative controls we tested PBS (E) or omitted addition of the primary antibody (F). Panels (A) and (B) show a greater number of lamina propria cells expressing IL-1β. 400X magnification.

Supplementary Fig. 4. iNOS expression in mice ileal loops inoculated with supernatants from strain ICC/45 and other *C. difficile* genotypes. The pictures shown represent the immunohistochemistry of tissue sections from mice injected with supernatants of (A) strain ICC-45, (B) NAP1/027, (C) NAP4/014-20, and (D) a non-toxigenic strains. As negative controls we tested PBS (E) or omitted addition of the primary antibody (F). Panels (A) and (B) show a higher expression of iNOS in epithelial cells compared to controls. 400X magnification.

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