The prevalence of *Clostridium difficile* infections has increased due to the emergence of epidemic variants from diverse genetic lineages. Here we describe the emergence of a novel variant during an outbreak in a Costa Rican hospital that was associated with severe clinical presentations. This *C. difficile* variant elicited higher white blood cell counts and caused disease in younger patients than did other strains isolated during the outbreak. Furthermore, it had a recurrence rate, a 30-day attributable disease rate, and disease severity as great as those of the epidemic strain NAP1. Pulsed-field gel electrophoresis genotyping indicated that the outbreak strains belong to a previously undescribed variant, designated NAPCR1. Whole-genome sequencing and ribotyping indicated that the NAPCR1 variant belongs to *C. difficile* ribotype 012 and sequence type 54, as does the reference strain 630. NAPCR1 strains are resistant to fluoroquinolones due to a mutation in *tcdC* that is characteristic of the epidemic, evolutionarily distinct, *C. difficile* NAP1 variant. NAPCR1 genomes contain 10% more predicted genes than strain 630, most of which are of hypothetical function and are present on phages and other mobile genetic elements. The increased virulence of NAPCR1 was confirmed by mortality rates in the hamster model and strong inflammatory responses induced by bacteria-free supernatants in the murine ligated loop model. However, NAPCR1 strains do not synthesize toxin A and toxin B at levels comparable to those in NAP1 strains. Our results suggest that the pathogenic potential of this emerging *C. difficile* variant is due to the acquisition of hypothetical functions associated with laterally acquired DNA.
induced by these NAP7 strains indicates that they might represent an emerging epidemic genotype; however, the molecular determinants associated with this behavior have not been addressed as thoroughly as for NAP1 strains. Other strains associated with severe disease have been recently described as well (18). In 2009 to 2010, a C. difficile outbreak occurred in a tertiary care hospital in Costa Rica. In a preliminary study performed with a partial collection of isolates from this outbreak, the presence of the NAP1 genotype was reported (19). Interestingly, a group of fluoroquinolone-resistant strains without NAP designation were also isolated (19). In this work, we report a group of C. difficile strains belonging to a previously undescribed NAP type with pathogenic potential similar to that of epidemic NAP1 strains. This emerging genotype is highly resistant to fluoroquinolones and possesses a deletion in tcdC similar to NAP1 strains; however, it lacks CDT and does not produce increased amounts of TcdA and TcdB. Together, these results describe the emergence of a C. difficile variant with high virulence potential.

MATERIALS AND METHODS

C. difficile isolation and strains. Stool samples positive for C. difficile toxins (Xpect Clostridium difficile toxin A/B test; Oxoid, Basingstoke, United Kingdom) that were collected during a CDI outbreak were processed. Samples were treated with 96% ethanol and inoculated onto cefoxitin-cycloserine-fructose agar (CCFA) plates (Oxoid, Basingstoke, United Kingdom), which were incubated for 5 days in an anaerobic chamber (Bactron II; Shel Lab, Cornelius, OR) under an atmosphere of 95% N2, 5% H2, and 5% CO2. Colonies were identified phenotypically (RapID 32A using the InstaGene reagent (Bio-Rad, Hercules, CA). Fragments of atpA, tpi, glyA, recA, and tcdB were amplified by PCR using primers and conditions reported previously (21).

Pulsed-field gel electrophoresis. The PFGE procedure used was derived from published protocols (4, 22). Briefly, bacteria from 6- to 8-h cultures in BHI broth were disrupted in lysis buffer. Agarose plugs were prepared by mixing equal volumes of bacterial suspensions and Seakem Gold agarose (Lonza, Basel, Switzerland) in 1× Tris-EDTA (TE) buffer (Sigma, Deisenhofen, Germany) containing SDS (Sigma). The plugs were incubated in a buffer composed of lysozyme, RNase A, and mutanolysin (Sigma). After overnight digestion with SmaI (Roche, Mannheim, Germany), DNA fragments were separated on 1% agarose gels in 0.5× Tris-borate-EDTA (TBE) buffer (Fermentas, St-Leon-Rot, Germany) containing 50 μM thiourea (Sigma), using a CHEF-DRII system (Bio-Rad). Images were analyzed with BioNumerics software (version 5.1; Applied Maths, Austin, TX) and macrorestriction patterns were compared to those deposited in the database of the National Microbiology Laboratory of the Public Health Agency of Canada (Winnipeg, Canada).

Antimicrobial susceptibility testing. MICs for ciprofloxacin, moxifloxacin, levofloxacin, clindamycin, metronidazole, rifampin, and vancomycin (Sigma) were determined using agar dilution, following the guidelines of the Clinical and Laboratory Standards Institute (23). Resistance breakpoints were set as follows: ciprofloxacin, >4 μg/ml; moxifloxacin, >4 μg/ml; levofloxacin, >4 μg/ml; clindamycin, >4 μg/ml; metronidazole, >16 μg/ml; rifampin, >32 μg/ml.

Clinical data. The study was a retrospective cohort study with patients with positive and confirmed C. difficile stool cultures. Each case was classified as nosocomial CDI or community-associated CDI according to criteria from the Infectious Diseases Society of America (IDSA) (24). CDI severity was categorized by applying the IDSA/Society for Healthcare Epidemiology of America (SHEA) criteria (24, 25) and criteria described by Zar et al. (26). Clinical data were extracted from patients’ medical records (27). The 30-day attributable mortality rate was calculated by considering patients with positive and confirmed C. difficile stool cultures who presented clinical signs and symptoms of CDI (temperature above 38°C, white blood cell count above 15,000 cell/mm3, or radiological evidence of pseudomembranous colitis) and whose death occurred within 30 days after the first diarrheal discharge. Categorical variables were analyzed by using logistic regression models, and risk factors were expressed in terms of odds ratios (ORs) with 95% confidence intervals (CIs). Two-tailed P values of 0.05 were used for significance. All statistical analyses were performed using IBM SPSS Statistics 20 software (IBM, Armonk, NY). Data collection was approved by the Ethics Committee of the San Juan de Dios Hospital (protocol CLOBI-HSJD-018-2009).

Whole-genome sequencing and sequence analysis. Whole-genome sequences of representative strains of each of the four NAPCR1 subtypes were obtained using multiplexed paired-end libraries and the sequencing-by-synthesis Illumina HiSeq platform. To this end, reads were assembled using Velvet (28), and contigs of >800 bp were scaffolded with SPSPACE (29) and ordered with ABACAS, using the C. difficile strain 630 genome as the reference (30). Gaps were filled using GapFiller (31), and the reads were mapped back to the assembly using SMALT (http://www.sanger.ac.uk/resources/software/small). Single-nucleotide polymorphisms (SNPs) were identified with RealPhy (version 1.07) (32) or with SAMTools (33). A dendrogram based on core SNPs was inferred via PhyML (34) and depicted using FigTree (http://tree.bio.ed.ac.uk/software/figtree). Deletions or truncations in tcdC, as well as mutations in gyrA and gyrB known to confer fluoroquinolone resistance, were identified using Artemis (35) and BLAST. Average nucleotide identities (ANIs) were computed using the genome-to-genome distance calculator at the German Collection of Microorganisms and Cell Cultures (36), and comparative genomic analyses were performed using the RAST server of the SEED framework (37).

Multilocus sequence typing and ribotyping. The sequence types (STs) of representative strains for each of the four NAPCR1 PFGE patterns were determined by using the multilocus sequence typing (MLST) 1.7 server maintained by the Center for Genomic Epidemiology at the Danish Technical University (38) and the classification scheme based on the genes adk, atpA, gpi, glyA, ddx, sodA, and recA, as proposed by Griffiths et al. (39). For ribotyping, primer sequences and reaction conditions were taken from the report by Biet et al. (40).

Hamster infection model. For the animal models, one representative strain was selected from each group isolated during the outbreak. All of the strains tested were resistant to clindamycin (NAPCR1 MIC, 256 μg/ml; NAP1 MIC, 16 μg/ml; NAP4 MIC, 9 μg/ml). Groups of 5 adult female Syrian Golden hamsters (150 to 180 g) were treated subcutaneously with 10 mg/kg clindamycin phosphate on day 0. On day 0, clindamycin-treated and nontreated control hamsters were inoculated, through the orogastric route, with 1,000 spores of the outbreak strains or the nontoxigenic ATCC 700057 strain suspended in Dulbecco’s modified Eagle medium (DMEM) (Sigma) (41). Hamsters were monitored at 12-h intervals for signs of C. difficile infection, such as diarrhea, and death. On days 1, 6, and 12, fecal pellets and intestinal contents of dead and surviving animals were processed for C. difficile isolation (42), and the resulting isolates were typed by PFGE to confirm the identity of the inoculated strain. All animal experiments were approved by the Animal Care and Use Committee of the Universidad de Costa Rica (protocols CICUA 01-12 and CICUA 07-13).

Murine ileal loop model. The strains were grown in TYT medium (3% Bacto tryptose, 2% yeast extract, and 0.1% thiglycollate [pH 6.8]) (Sigma) for the indicated times. Bacteria were removed by centrifugation at 20,000 × g for 30 min, and supernatants were passed through 0.2-μm filters.

Male Swiss mice (20 to 25 g) were fasted overnight and anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg) (Kö nig, São Paulo, Brazil). Through a midline laparotomy, a 4-cm ileal loop was ligated and injected with 0.3 ml of supernatants or the corresponding control solu-
acetic acid, proteinase K (Fermentas; Fisher Scientific, Pittsburgh, PA), and tetracycline that caused 50% cell rounding in the monolayers (i.e., 50% CPE). Cytotoxicity was expressed as the inverse of the dilution of the supernatants. TcdB-specific antiserum (TechLab, Blacksburg, VA) was used to neutralize the effect of the toxin. Cytotoxicity was expressed as the inverse of the dilution of the supernatants that caused 50% cell rounding in the monolayers (i.e., 50% CPE).

**Cytotoxicity assays.** Ten-fold dilutions of the supernatants were added to HeLa cell monolayers grown in DMEM supplemented with 5% fetal bovine serum. The cells were monitored for the appearance of cytopathic effect (CPE) by optical microscopy. TcdB-specific antiserum (TechLab, Blacksburg, VA) was used to neutralize the effect of the toxin. Cytotoxicity was expressed as the inverse of the dilution of the supernatants that caused 50% cell rounding in the monolayers (i.e., 50% CPE).

**Toxin quantitation.** The toxins were quantified in the same strains as used for the animal models. The amounts of toxins secreted by the strains were quantified by Western blotting. Proteins from bacteria-free supernatants were separated by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were probed with monoclonal anti-TcdA (TTC8) or anti-TcdB (2CV) antibodies (tgBiOMICS, Mainz, Germany) (46). Chemiluminescence signals emitted after addition of a goat anti-mouse IgG-horseradish peroxidase conjugate (Invitrogen; Life Technologies, Carlsbad, CA) and the LumiGLO Plus Western blotting substrate (Roche) were recorded with a Chemidoc XR system (Bio-Rad).

Transcripts of tcdA and tcdB were quantified by quantitative reverse transcription (qRT)-PCR. The different strains were grown on TYT medium, and 1 x 10^6 cells were processed for RNA extraction. Bacteria were pelleted by centrifugation at 5,000 x g and lysed with lysostaphin (Sigma), acetic acid, and protease K (Fermentas; Fisher Scientific, Pittsburgh, PA), and SDS (Sigma) (47). RNA was isolated with the RNeasy Midi kit (Qiagen, Hilden, Germany) and treated with DNase I Turbo (Ambion; Life Technologies, Austin, TX). Two micrograms of RNA was reverse transcribed to cDNA using RevertAid transcriptase (Fermentas). The amplification conditions were as reported previously (48). The relative expression of genes was calculated with the threshold cycle (ΔΔCt) method, using the rpoA transcript as an endogenous control.

**Nucleotide sequence accession numbers.** The sequence information from this whole-genome shotgun project has been submitted to DDBJ/EMBL/GenBank under accession numbers JXCP00000000, JXBQ00000000, JXBQ00000000, JXBR00000000, and JXBS00000000, as part of BioProject PRJNA264745.

**RESULTS**

**Isolation and molecular characterization of an emerging strain.** During a C. difficile outbreak in a tertiary care hospital in Costa Rica (Fig. 1), 57 strains were isolated. These strains were assigned to 16 SmaI macrorestriction patterns using PFGE (Fig. 2A). Of those patterns, 7 belonged to previously described genotypes (NAP1, NAP2, NAP4, NAP6, and NAP9), whereas 9 did not match an existing NAP designation. Four of the unclassified SmaI PFGE patterns were frequently isolated and were preliminarily designated NAP1R (Fig. 2A). Future allocation of this genotype into the standard established NAP nomenclature requires the appearance of additional C. difficile strains displaying related macrorestriction patterns in other geographical locations. NAP1 and NAP1R strains accounted for the majority of isolates (45% and 31%, respectively). All of these strains were positive for tcdA, tcdB, and tcdC. In addition, NAP1R strains showed an 18-bp deletion in tcdC, as in NAP1 strains, but only NAP1 strains had a single-base-pair deletion at position 117.

**NAP1R and NAP1 strains are associated with increased disease severity.** To characterize the clinical and epidemiological spectra of CDIs produced by the different genotypes, patient data were classified into three groups, i.e., patients infected with NAP1 strains (n = 26), patients infected with NAP1R strains (n = 18), and patients infected with strains of other genotypes (n = 13). The average ages of patients were significantly different (Table 1); whereas NAP1R strains affected younger patients, NAP1 strains and strains of other genotypes affected older individuals. Patients infected with NAP1R strains presented higher white blood cell counts, and most of them were male. In general, there were no significant differences in the distributions of comorbidities. However, noticeable numbers of infections with NAP1 and NAP1R strains were associated with trauma (Table 2).

CDIs with NAP1R (100%) and NAP1 (96%) strains were hospital-acquired, whereas 31% of the cases caused by other genotypes were community-associated CDIs. Patients undergoing fluoroquinolone therapy were 10 or 14 times more likely to develop infections caused by NAP1R or NAP1 strains, respectively, than infections caused by other genotypes (NAP1R versus other genotypes, P = 0.04 [OR, 9.6]; NAP1 versus other genotypes, P = 0.006 [OR, 14]) (Table 2). Infections with NAP1R and NAP1 strains were more likely to be associated with increased disease severity, according to the IDSA/SHEA criteria (NAP1R versus other genotypes, P = 0.03 [OR, 6.87 [95% CI, 1.17 to 40.38]]) and the criteria described by Zar et al. (26) (NAP1R versus other genotypes, P = 0.03 [OR, 5.83 [95% CI, 1.22 to 27.99]]) (Table 2). Recurrence was 10 and 9 times more likely in patients infected with NAP1R and NAP1 strains, respectively, than in patients infected with other genotypes (NAP1R versus NAP1 strains, P = 0.04 [OR, 9.6]; NAP1 versus other genotypes, P = 0.03 [OR, 8.8]) (Table 2). Similarly, the 30-day attributable mortality rates for NAP1R and NAP1 strains were significantly higher (17% and 27%, respectively) than that
for strains of other genotypes ($P = 0.05$ and $P = 0.03$, respectively) (Table 2).

**Comparative genomic analysis of NAP<sub>CR1</sub> strains.** To further study the NAP<sub>CR1</sub> variant, we performed whole-genome sequencing and comparative genomic analysis. All of the NAP<sub>CR1</sub> strains are very closely related, as indicated by the finding of only 101 core SNPs in the ~4.5-Mb genomes (Fig. 2B). When we compared NAP<sub>CR1</sub> genomes to reference genomes from common *C. difficile* genotypes, we found that the NAP<sub>CR1</sub> strains shared only 101 core SNPs, indicating a high degree of genomic similarity.

![Molecular characterization of C. difficile isolates.](image-url)
strains, the NAPCR1 strains were not related to NAP1 lineages (68,413 core SNPs). Instead, average nucleotide identity (ANI) of 99% and 405 SNPs distinguished the core genomes of NAPCR1 and C. difficile strain 630. By ribotyping we determined that NAPCR1 belongs to ribotype 012, and by MLST we determined that this strain belongs to ST54 (data not shown). Strain 630 belongs to the same typing groups, which confirms the close relationship with NAPCR1.

NAPCR1 strains have more laterally acquired DNA than close relatives. NAPCR1 has about 6% more DNA and 10% more predicted proteins than C. difficile strain 630 (4,549,499 bp and 4,201 proteins versus 4,290,252 bp and 3,819 proteins). Compared to C. difficile strain 630, NAPCR1, has almost twice as many functions from the category of phages, prophages, transposable elements, and plasmids (see Table S1 in the supplemental material). Further functional differences were mostly related to DNA/RNA metabolism and regulation and cell signaling (see Table S1 in the supplemental material). Metabolic reconstruction of the NAPCR1 and C. difficile strain 630 genomes revealed that the NAPCR1 genotype has genes from 14 different categories not present in C. difficile strain 630. Six of these categories are associated with phages, and an additional one has to do with antibiotic resistance. In contrast, NAPCR1 lacks genes related to chorismate synthesis, Ton and Tol transport, phage DNA synthesis, and phage-packaging machinery. NAPCR1 and C. difficile strain 630 have 338 and 161 unique genes, respectively (see Tables S2 and S3 in the supplemental material). Almost all unique NAPCR1 sequences encode hypothetical proteins and cluster in contigs carrying phage genes or, to a minor extent, antibiotic resistance genes (see Table S2 in the supplemental material).

NAPCR1 strains display a virulent phenotype. The pathogenic potential of NAPCR1 strains was compared to that of other geno-

### Table 1: Characteristics of patients with CDIs caused by different genotypes

<table>
<thead>
<tr>
<th>Variable*</th>
<th>NAP1 (n = 26)</th>
<th>NAPCR1 (n = 18)</th>
<th>Other genotypes (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD) (yr)</td>
<td>70 ± 22</td>
<td>48 ± 30</td>
<td>60 ± 35</td>
</tr>
<tr>
<td>Hospital stay before onset of diarrhea (mean ± SD) (days)</td>
<td>11 ± 20</td>
<td>24 ± 47</td>
<td>12 ± 32</td>
</tr>
</tbody>
</table>

*SD, standard deviation.

### Table 2: Univariate analysis of risk factors and outcomes of CDIs caused by different genotypes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Proportion (%)</th>
<th>P (OR [95% CI])*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>57.7</td>
<td>1.0 (no OR)*</td>
</tr>
<tr>
<td>Nosocomial CDI</td>
<td>96.1</td>
<td>0.04 (6.85 [1.10-42.75])</td>
</tr>
<tr>
<td>Underlying disease</td>
<td>0.04 (5.86 [1.11-90.34])</td>
<td>1.0 (0.85 [0.22-3.32])</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>76.9</td>
<td>1.0 (no OR)*</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>7.7</td>
<td>0.04 (9.6 [1.02-90.34])</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease</td>
<td>7.7</td>
<td>0.04 (9 [1.0-84.49])</td>
</tr>
<tr>
<td>Malignancy</td>
<td>30.8</td>
<td>1.0 (1.11 [1.09-113.06])</td>
</tr>
<tr>
<td>Trauma</td>
<td>3.8</td>
<td>1.0 (1.0 [0.13-12.44])</td>
</tr>
<tr>
<td>Pharmacotherapy</td>
<td>38.5</td>
<td>0.09 (0.04-0.12)</td>
</tr>
<tr>
<td>Antibiotic use within 8 wk prior to CDI</td>
<td>53.8</td>
<td>0.76 (1.25 [0.38-4.18])</td>
</tr>
<tr>
<td>Fluoroquinolone exposure within 8 wk prior to CDI</td>
<td>46.1</td>
<td>0.76 (0.68 [0.20-2.30])</td>
</tr>
<tr>
<td>Gastric acid suppressors</td>
<td>96.1</td>
<td>1.0 (0.68 [04-11.63])</td>
</tr>
<tr>
<td>Clinical features</td>
<td>53.8</td>
<td>0.28 (5.1 [0.46-55.89])</td>
</tr>
<tr>
<td>White blood cell count of &gt;15,000 cells/µl</td>
<td>38.5</td>
<td>0.09 (7.5 [0.69-80.95])</td>
</tr>
<tr>
<td>Albumin level of &lt;2.5 mg/dl</td>
<td>61.5</td>
<td>1.0 (1.25 [0.38-4.18])</td>
</tr>
<tr>
<td>Fever of &gt;38°C</td>
<td>50</td>
<td>0.76 (1.25 [0.37-4.17])</td>
</tr>
<tr>
<td>Severe disease according to IDSA/SHEA criteria</td>
<td>69.2</td>
<td>0.03 (6.87 [1.17-40.38])</td>
</tr>
<tr>
<td>Severe disease according to criteria of Zar et al. (26)</td>
<td>38.5</td>
<td>0.03 (5.50 [1.01-29.85])</td>
</tr>
<tr>
<td>Recurrence</td>
<td>30.8</td>
<td>0.03 (5.06 [1.20-21.42])</td>
</tr>
<tr>
<td>30-day all-cause death</td>
<td>30.8</td>
<td>0.74 (1.43 [0.40-5.06])</td>
</tr>
<tr>
<td>30-day attributable death</td>
<td>26.9</td>
<td>0.55 (0.32 [0.07-1.39])</td>
</tr>
</tbody>
</table>

*P values were calculated using Fisher’s exact test.

Statistically significant (P ≤ 0.05).

OR could not be calculated because one of the proportions was 0% or 100%.
types isolated in the outbreak by using two animal models, i.e., the hamster-spore infection model and murine ligated ileal loops inoculated with bacteria-free supernatant. In order to determine the rates of deaths induced by each genotype, clindamycin-treated hamsters were infected with spores. NAP\textsubscript{CR1}, NAP1, NAP4, and nontoxigenic strains colonized 100% of the hamsters within 6 days. The survival rates of hamsters inoculated with NAP\textsubscript{CR1} and NAP1 spores declined rapidly, with the groups reaching 40% survival at day 5 and day 3, respectively (Fig. 3). In contrast, the survival rate for hamsters inoculated with NAP4 spores was 80% at 12 days after inoculation (Fig. 3). All animals inoculated with spores from nontoxigenic strains survived the duration of the experiments (Fig. 3). Inoculation of non-antibiotic-treated hamsters with spores from all of the genotype groups failed to result in colonization.

In the ligated ileal loop model, the NAP1 supernatant induced strong inflammatory reactions, measured as the normalized weights of the ligated ileal loops (100 ± 15 mg/cm). The NAP\textsubscript{CR1} supernatant induced a less severe reaction (66 ± 10 mg/cm) than that induced by the NAP1 strain; however, the response elicited was stronger than that induced by the NAP4 supernatant (54 ± 6 mg/cm). Histological analyses indicated that the NAP\textsubscript{CR1} and NAP1 supernatants induced greater inflammatory cell infiltration and edema than did the NAP4 supernatants (Fig. 4). Only the NAP1 supernatant induced intense mucosal disruption with epithelial damage (Fig. 4).

We measured the concentration of myeloperoxidase (MPO)
activity as an indicator of tissue neutrophil infiltration and the levels of IL-1β, IL-6, and TNF-α to indicate immune activation at the ileal tissue level. NAP4CR1 and NAP1 supernatants caused statistically significant increases in MPO activity, in contrast to the NAP4 supernatant, which elicited a reaction similar to that observed with a nontoxicogenic control (see Fig. S1 in the supplemental material). IL-6 and TNF-α levels were strongly induced in ileal tissue by NAP4CR1 and NAP1 supernatants. Again, the NAP4 supernatant induced a reaction similar to that observed with the nontoxicogenic control (see Fig. S1 in the supplemental material). IL-1β expression was highly induced by the NAP1 supernatant, compared to the other C. difficile strains (see Fig. S1 in the supplemental material).

NAP4CR1 strains are highly resistant to fluoroquinolones.
NAP4CR1 and NAP1 strains were resistant to levofloxacin and levofloxacin, whereas almost all of the other genotypes were susceptible to these antibiotics (see Fig. S2 in the supplemental material). In addition, the NAP4CR1 but not NAP1 strains were also resistant to clindamycin and rifampin (see Fig. S2 in the supplemental material). Since fluoroquinolone resistance in C. difficile has been attributed to point mutations in either gyrA or gyrB, we sequenced those genes in selected strains from the outbreak (49). As reported previously, NAP1 strains presented the Thr82-to-Ile amino acid substitution in GyrA (50, 51). Among fluoroquinolone-susceptible control isolates (NAP4 and NAP6), no mutations were detected in either gyrA or gyrB.

NAP4CR1 strains do not produce increased amounts of toxins.
We compared the ability to produce and to secrete toxins in selected strains isolated during the outbreak by measuring toxin activity, TcdA and TcdB protein levels, and expression of tcdA and tcdB transcripts. NAP1 strains consistently gave higher cytotoxic titers than did NAP4CR1 and NAP4 strains (Fig. 5A). Supernatants from each group of strains were collected at different times during the growth cycle, and the amounts of toxin were determined by Western blotting. TcdA was detected in NAP1 supernatants within the first 4 h, and concentrations increased steadily up to 24 h (Fig. 5B). The amounts of TcdA were lower in NAP4CR1 and NAP4 supernatants at all times, being barely detectable at 8 h and increasing up to 48 h (Fig. 5B). TcdB was detected in NAP1 supernatants at 8 h, and its concentration peaked at 24 h. In contrast, TcdB was detectable in supernatants from NAP4CR1 and NAP4 strains only at 24 h, at lower concentrations (Fig. 5B). The tcdA and tcdB mRNAs were quantified by real-time PCR, and the levels of both transcripts were higher in NAP1 strains than in NAP4CR1 and NAP4 strains at all times tested (Fig. 5C).

DISCUSSION
The incidence and severity of CDIs are increasing throughout the world (52, 53), a phenomenon that is partly due to the emergence of epidemic C. difficile strains (13, 14, 18, 54, 55). Here we described the emergence of a C. difficile strain with genetic, clinical, and virulence features that resemble those of NAP1 strains but within a C. difficile lineage, ribotype 12/ST54, for which no epidemic strains have been reported previously.

The NAP4CR1 genotype displays a more aggressive phenotype both in clinically infected patients and in animal models. Patients whose CDIs were caused by NAP4CR1 strains were younger than those affected by NAP1 and other genotypes and interestingly, as with the highly virulent NAP1 strains, a significant percentage of NAP4CR1 cases were associated with trauma as a risk factor. These two epidemiological characteristics depart from the classic profile for patients affected by C. difficile, which includes age of >75 years and chronic debilitating diseases as risk factors. In addition, patients infected by NAP4CR1 presented recurrence rates and 30-day attributable mortality rates as high as those presented by patients infected by NAP1. Furthermore, a majority of patients affected by NAP4CR1 presented white blood cell counts higher than 15,000 cells/µl, supporting the proinflammatory nature of the response elicited by this particular strain (see below). Thus, the clinical picture induced by NAP4CR1 strains, as measured using different standardized clinical criteria, is as severe as that induced by strains
of the epidemic NAP1 genotype. The NAP_{CR1} strain was isolated from approximately one-third of the patients involved in the outbreak described in this report. The epidemic genotype, NAP1, was detected in a similar percentage, whereas other genotypes were less frequently represented. It is important to note that in this study we worked with toxin-positive samples; considering that NAP_{CR1} strains do not produce increased amounts of toxins, it is possible that some milder cases involving this group of strains were missed and that the overall prevalence of NAP_{CR1} is even higher than that reported here.

The more virulent phenotype of NAP_{CR1} was also demonstrated with animals challenged orally with spores; the NAP_{CR1} strain was as virulent as the NAP1 strain in terms of the ability to decrease the survival rate for clindamycin-treated hamsters. In contrast, in this model the NAP1 strain has consistently displayed increased lethality versus strain 630 (41, 56, 57). Considering the close phylogenetic relationship between NAP_{CR1} and strain 630 and the fact that both strains are resistant to clindamycin, the different behavior in the hamster model indicates that the latter has acquired virulence factors that increase its pathogenicity. These observations also indicate a positive correlation between severe CDI outcomes in humans and increased lethality of epidemic strains in animal models, thus demonstrating the increased virulence of NAP_{CR1}.

A factor consistently associated with the selection and spread of NAP1 strains is resistance to fluoroquinolones (6, 49, 50), and it is well documented that restriction in the usage of these antibiotics results in decreases of CDIs (58, 59). In this work, we report that fluoroquinolone resistance is shared by NAP_{CR1} and NAP1 strains. The NAP_{CR1} strains harbor the same mutation in gyrA as reported for fluoroquinolone-resistant NAP1 strains, suggesting that the two genotypes share the same mechanism of resistance. This mutation has not been previously reported in ribotype 12/ST34 (60, 61). This observation reflects the successful nature of this mutation in conferring fluoroquinolone resistance to \textit{C. difficile} in response to the selection pressure imposed by the use of this family of antibiotics. It is clear that, while fluoroquinolone resistance is not a molecular determinant of pathogenicity, the high level of antibiotic resistance introduces a major selection force that favors the dissemination of epidemic and endemic strains and thus is a procolonization factor (16).

The pathogenic phenotype of NAP1 strains has been attributed to increased production of TcdA and TcdB (9, 62). Some studies have suggested that this characteristic is related to deletions in the \textit{tdcC} gene (8, 63). Thus, we hypothesized that the aggressive phenotype demonstrated by the NAP_{CR1} strain, which also presents an 18-bp deletion in \textit{tdcC}, would depend on increased toxin production. This was clearly not the case, however, since the levels of TcdA and TcdB produced by the NAP_{CR1} strain were significantly lower than the levels of toxins produced by NAP1 strains. This lack of correlation between \textit{tdcC} deletions and increased toxin production has been documented previously (11, 64). Despite the presence of the 18-bp deletion in \textit{tdcC}, the NAP_{CR1} genotype does not exhibit the frameshift mutation at position 117, which probably has a greater impact on the functionality of TcdC as a negative regulator of TcdA and TcdB expression (63). Thus, molecular factors other than increased levels of toxins could account for the increased virulence observed for the NAP_{CR1} strain. These factors could involve metabolic and/or pathogenic adaptations that allow the bacteria to colonize the intestines of affected patients more efficiently. In the murine ligated loop model, we found that the inflammatory response elicited by NAP_{CR1} supernatants was almost as strong as that induced by NAP1 supernatants, despite the remarkable differences in TcdA and TcdB concentrations between the two strains. Thus, we present evidence that the emerging NAP_{CR1} genotype is able to induce inflammatory reactions (neutrophil recruitment and cytokine induction) and epithelial damage usually attributed to TcdA and TcdB. This capacity may be associated with other virulence factors that have not yet been described for NAP_{CR1} and that would be responsible for the aggressive pathological response. This hypothesis is in agreement with recent reports demonstrating several effects of \textit{C. difficile} on host immunity that are toxin independent (65–69). Since the NAP_{CR1} strain belongs to the ribotype 012/ST54 group, for which no epidemic strains have been reported previously, its increased virulence could reside in the additional genomic content found in this genotype, in comparison with \textit{C. difficile} strain 630. It is difficult at this point to assign the virulent phenotype to a particular set of genes, due to the large number and hypothetical nature of these additional open reading frames. However, the abundance and diversity of prophages found in the emerging NAP_{CR1} genotype could play a role in the increased virulence of this strain, since these genetic elements have been found previously to be involved in the regulation of virulence-associated genes (70–73). In this scenario, new virulent strains may arise through the acquisition of foreign DNA, with the ability to modulate immune responses, to tolerate antibiotics, and to regulate expression of virulence traits.

In conclusion, we described an emerging strain that possesses increased virulence potential due to the acquisition of laterally acquired genes and its ability to induce an exacerbated inflammatory response in the gastrointestinal mucosa, through currently unknown mechanisms. The emergence of strains with increased virulence is of importance in the surveillance of \textit{C. difficile} outbreaks associated with both endemic and epidemic strains.

**ACKNOWLEDGMENTS**

This work was supported by the Ministry of Science, Technology, and Telecommunications of the Republic of Costa Rica, the National Rector’s Council, Costa Rica, through grant 803-B1-654, the Postgraduate Studies System and Vice-rectory for Research of the University of Costa Rica through grant 803-B1-602, the National Council of Science and Technology of Costa Rica through FORINVES grant FV-0004-13, FUNCAP (Ceará State, Brazil) through Programa de Cooperação Internacional grant 130621650, and the Wellcome Trust (grants 098051 and 086418).

We thank Pablo Vargas for technical assistance and the staff of the clinical laboratory of the San Juan de Dios Hospital for the clinical samples.

No potential conflicts of interest are declared.

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