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Emergence of Clinical *Clostridioides difficile* Isolates With Decreased Susceptibility to Vancomycin

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Background. *Clostridioides difficile* infection (CDI) is a leading cause of hospital-associated antibiotic-related diarrhea and deaths worldwide. Vancomycin is one of the few antibiotics recommended for both nonsevere and severe CDI cases. We sought to determine whether vancomycin nonsusceptible *C. difficile* strains are circulating in the patient population.

Methods. Stool samples from patients with CDI were collected from 438 and 98 patients at a large university hospital in Houston, Texas, and Nairobi, Kenya, respectively. The stools were examined for the presence of vancomycin and metronidazole nonsusceptible *C. difficile* using broth dilution culture, Etest (BioMérieux, France), polymerase chain reaction (PCR), whole-genome sequencing, and *in vivo* testing in a CDI mouse model.

Results. Of the Houston stool samples, 114/438 (26%) had vancomycin nonsusceptible *C. difficile* isolates and 128/438 (29%) were metronidazole nonsusceptible. Similarly, 66 out of 98 (67%) and 83/98 (85%) of the Nairobi patients harbored vancomycin and metronidazole nonsusceptible isolates, respectively. Vancomycin treatment of a CDI mouse model infected with a vancomycin nonsusceptible isolate failed to eradicate the infection. Whole-genome sequencing analyses did not identify *vanA* genes, suggesting a different mechanism of resistance.

Conclusions. *C. difficile* strains exhibiting reduced susceptibility to vancomycin are currently circulating in patient populations. The spread of strains resistance to vancomycin, a first-line antibiotic for CDI, poses a serious therapeutic challenge. Routine susceptibility testing may be necessary.

Keywords. *Clostridioides difficile*; *Clostridioides difficile* infections; antibiotic resistance; high-level vancomycin resistance; *C. difficile* vancomycin susceptibility.

The past decade has recorded a remarkable increase in *Clostridioides difficile* infections (CDIs), with recurrence becoming a major problem in clinical settings. Indeed, antibiotic-based treatment of CDI has been ineffective in as many as 25% of the cases, resulting in recurrence of the infection [1]. Treatment of CDI is challenging because of sporulation and emergence of strains resistant to multiple antibiotics. The Infectious Diseases Society of America (IDSA) and the Society for Healthcare Epidemiology of America (SHEA) support the use of oral vancomycin for both nonsevere and severe CDI cases [2].

Nine vancomycin-resistance gene clusters have been reported, mostly in *Enterococcus* spp. and other vancomycin-resistant bacteria, namely *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN* [3–6]. The *vanA* and *vanB* clusters mediate high-level vancomycin resistance. *vanA* is part of an operon consisting of regulatory genes (*vanRS*) and genes that encode metabolic-associated proteins (*vanHAXY*) to perform 2 functions: (1) synthesis of D-lactate for incorporation into peptidoglycan precursors (*vanHA*) and (2) destruction of "normal" D-alanine ending precursors (*vanXY*) [7]. The full expression of the cluster results in inducible remodeling of peptidoglycan precursors in which the usual D-alanine–D-alanine termini of lipid II precursors (the target for vancomycin) is replaced with D-alanine– D-lactate, decreasing the binding affinity of the drug more than 1000-fold [7]. A second vancomycin-resistance pathway involves replacing the terminal D-alanine with D-serine (VanCEG types) [4, 7–9]. This modification also reduces the affinity of vancomycin, albeit much less than that of D-lactate (only ~20-fold) [7]. These genes are usually carried on transposable elements inserted either on plasmids or on the chromosome [4, 7–9]. Acquisition of any of these clusters in *C. difficile*, particularly those encoding high-level resistance to vancomycin, would markedly affect the effectiveness of this antibiotic and presents a major challenge to the already complicated CDI treatment options available.

Due to the common occurrence of recurrent post-vancomycin-treatment of CDI, we sought to investigate whether vancomycin-resistant *C. difficile* isolates are circulating in the

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patient population by examining stools of patients with CDI from Houston, Texas, and Nairobi, Kenya. Here, we show that *C. difficile* strains exhibiting reduced susceptibility to vancomycin are emerging in these 2 different geographical areas and provide *in vivo* data suggesting that resistance to vancomycin may lead to therapeutic failure.

METHODS

Stool Samples

Clinical stool samples were obtained from inpatients who presented with diarrhea at a large university hospital in Houston, Texas, from 2012 to 2017 and from a national hospital in Nairobi, Kenya, from May to July 2017. This study was approved by the institutional review boards (IRBs) of The University of Texas Health Science Center at Houston and the Ethics Review Board of Kenyatta National Hospital and University of Nairobi (KNH/UoN-ERC). In Houston, patients positive for *C. difficile* toxin were consecutively enrolled. All stool samples were initially tested by real-time polymerase chain reaction (PCR) for the toxin genes and classified as *C. difficile* positive by the Medical Microbiology Laboratory at the hospital. In Nairobi, patients who reported to the Kenyatta National Hospital with diarrhea were sequentially enrolled and stool samples were collected for analysis. The IRB approval stipulated that all of the stool samples be de-identified and, as a result, no patient information could be included in the current study.

Screening for Metronidazole and Vancomycin Nonsusceptible *Clostridioides difficile*

The presence of *C. difficile* in the stool samples was determined using toxigenic *C. difficile* culture and PCR [10–12]. The *C. difficile* culture medium (CDPA) is composed of brain heart infusion (BHI) broth (Becton Dickinson, Cockeysville, MD) (37 g), agar (14 g), defibrinated horse blood (7%) (Quad Five, Ryegate, MT), 150 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Zymo Research, Irvine, CA), *p*-cresol (0.025%), 0.1% sodium taurocholate (Sigma-Aldrich, St Louis, MO), D-cycloserine (500 μg/mL), and cefoxitin (16 μg/mL) (Fisher Scientific, Hampton, NH). To identify stool samples containing nonsusceptible *C. difficile* isolates, samples were plated on CDPA only and CDPA containing either 8 μg/mL of metronidazole or 4 μg/mL of vancomycin based on breakpoint concentrations published by the Clinical and Laboratory Standards Institute (CLSI) [13–16]. Using a sterilized loop, each stool sample was spread on CDPA medium and incubated anaerobically at 37°C for 48 hours. Anaerobic conditions were maintained by establishing an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 in a Bactron 600 anaerobic chamber (Sheldon Manufacturing, Inc, Cornelius, OR). A total of 536 stool samples were tested (438 from Houston and 98 from Kenya). Colonies from respective stool samples were enumerated and single colonies were selected from each plate (5–10 colonies per patient stool) and further purified by streaking on nonselective BHI agar, followed by culture in CDPA containing 4 μg/mL of vancomycin. Pure colonies were also tested for enterococcal contamination by plating on Slanetz and Bartley medium (Oxoid, Hampshire, UK).

DNA Isolation and Polymerase Chain Reaction Confirmation

DNA was extracted from the pellet of each isolate using the GenElute Bacteria Genomic DNA Kit (Sigma-Aldrich, St Louis, MO). To confirm the isolates to be *C. difficile*, PCR was performed using primers specific for toxins A and B genes (*tcdA*, *tcdB*), *tcdC*, *agrD* gene, and the 16S ribosomal RNA (rRNA) gene (control), as previously published [10, 11, 17–24].

Toxin Assays

Clostridioides difficile toxins A and B present in 48-hour cultures from respective isolate supernatants were detected using the Cdifftox activity assay for toxin activity [25] and *C. difficile* TOX A/B II enzyme-linked immunosorbent assay (ELISA) test (TechLab, Blacksburg, VA) for toxin production. The Cdifftox activity assay was performed, as previously reported [10, 11, 24, 26]. For the ELISA test, 200 μL of culture supernatants was tested, according to the manufacturer's instructions.

Minimum Inhibitory Concentration Determination

Nonsusceptible isolates from each patient were subjected to vancomycin minimum inhibitory concentration (MIC) determination using Etest (BioMérieux, France) and confirmed with the broth microdilution method [27, 28]. For the Etest, overnight cultures were diluted in sterile 1× phosphate buffered saline (PBS) and spread onto pre-reduced Mueller Hinton agar plates using a sterilized cotton swab. Etest strips impregnated with vancomycin (0.16–256 μg/mL) were placed onto each plate using sterilized forceps and incubated anaerobically at 37°C for 24 hours. The MICs were determined based on the zone of inhibition. For the broth microdilution method [27, 28], 20–30 μL of overnight cultures (optical density [O.D.] 600 nm, 0.5–0.9) of each isolate was added in triplicate to a sterile 96-well plate containing 290 μL pre-reduced Mueller Hinton broth containing vancomycin or teicoplanin at concentrations ranging from 0 to 1024 μg/mL. The culture was incubated anaerobically at 37°C for 24 hours and O.D. 600 nm was measured.

Whole-Genome Sequencing

Whole-genome sequencing was performed on 10 isolates with vancomycin MICs of 4 μg/mL or greater (3 isolates), 16–32 μg/mL (4 isolates), and more than 32 μg/mL (3 isolates). DNA was extracted using the Qiagen QIAmp mini kit (Qiagen, Germantown, MD) following the manufacturer's protocol. DNA was prepared for short-read sequencing using the Illumina Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA) and was sequenced on an Illumina MiSeq using the 2 × 300 v3 chemistry. Additionally,

DNA was prepared for long-read sequencing using the Oxford Nanopore Rapid Barcoding Kit (SQK-RBK004; Oxford Nanopore, Cambridge, UK) and was sequenced on an Oxford Nanopore GridION X5 using the 9.4 chemistry. A consensus assembly was generated with the Canu assembler [29] and polished using Racon with the high-accuracy short-read data [30]. This assembly was then circularized using berokka (https://github.com/tseemann/berokka) and polished an additional 3 times with the high-accuracy short-read data using Racon. The sequencing was performed at the Center for Antimicrobial Resistance and Microbial Genomics, UTHealth, McGovern School of Medicine (Houston, TX), and Novogene Corporation (Sacramento, CA). The raw sequences from Novogene Corporation were assembled using SPAdes [31] and annotated on the RAST server [32, 33]. All of the sequences have been deposited in the NCBI (National Center for Biotechnology Information) database and are available under BioProject number PRJNA631049.

Clostridioides difficile Infection Murine Model

Both female and male 7-week-old C57BL/6 mice (n = 12 in each group) were administered a cocktail of antibiotics for 7 days in their drinking water. The antibiotics included kanamycin (40 mg/kg), gentamicin (3.5 mg/kg), colistin (4.2 mg/kg), metronidazole (21.5 mg/kg), and vancomycin (4.5 mg/kg) [24, 34]. One day after the antibiotic treatment, mice were administered 1 mg/kg clindamycin by intraperitoneal injection. Twenty-four hours following the clindamycin treatment, mice were infected with 10^6 spores of either vancomycin susceptible R20291 strain or a vancomycin nonsusceptible isolate (from a Houston patient with MIC >16 μg/mL) in a suspension of 250 μL PBS by oral gavage. The infected mice were treated twice daily by oral gavage with and without vancomycin (20 mg/kg) beginning 24 hours postinfection for 4 days. The mice were observed twice daily for 14 days postinfection and scored based on 3 main endpoint parameters as previously reported [24]: (1) diarrhea, hunched posture, and physical appearance; (2) movement and response to external stimulus; and (3) weight loss. Animal scoring was performed independently by 2 individuals in a blinded fashion.

Pellets from mice collected on day 4 postinfection were also tested for the presence of toxins A and B using the Wampole *C. difficile* TOX A/B II assay. Briefly, 10–20 pellets were collected from each mouse and suspended in 250 μL of PBS. The ELISA was performed using the instructions provided by the manufacturer. The pellets were also tested for the presence of *C. difficile* cells by colony-forming unit counts.

Data Analyses

Data were analyzed and plotted using GraphPad Prism 7.05 (San Diego, CA). Student's *t* test was used to compare differences between samples. A Kaplan-Meier curve and the Gehan-Breslow-Wilcoxon test were used to analyze survival of the animals. In all cases, statistical significance was defined as having a p value of less than .05.

RESULTS

We examined clinical stools from 438 consecutively enrolled patients with acute diarrhea hospitalized in a major teaching hospital in Houston, Texas, for the presence of metronidazole and vancomycin nonsusceptible *C. difficile* isolates. An additional 98 patients with diarrhea from a major university hospital in Nairobi, Kenya, were included. In order to determine the number of patients infected with nonsusceptible isolates, their stools were initially cultured on *C. difficile*–specific medium containing either 8 μg/mL metronidazole or 4 μg/mL vancomycin (CLSI cutoff values) [13–16]. Of the stools obtained from Houston patients, 114 of 438 (26%) grew *C. difficile* isolates on vancomycin plates, 128 of 438 (29%) on metronidazole plates, and 97 of 438 (22%) grew on both metronidazole and vancomycin plates (Figure 1A). On the other hand, in the Kenyan hospital, 66 of 98 (67%) of the stools grew *C. difficile* isolates on vancomycin plates, 83 of 98 (85%) on metronidazole plates, and 57 of 98 (58%) grew on both metronidazole and vancomycin plates (Figure 1B). Pearson's test of association between nonsusceptibility and location showed a higher tendency for the Kenyan patients with CDI to be infected by both metronidazole and vancomycin nonsusceptible strains than patients from Texas ($p \leq .001$).

Pure colonies of the nonsusceptible isolates were selected and further confirmed by PCR amplification of genes (*tcdA*, *tcdB*, *tcdC*, *agrD*, and 16S rRNA) specific to *C. difficile*. All of the isolates were positive for the *C. difficile* genes examined and produced toxins A and B. Moreover, whole-genome sequencing of 10 isolates confirmed the presence of these genes. One isolate was randomly selected from each patient and vancomycin MIC was determined. The selected isolates from both patient populations showed reduced susceptibility to vancomycin, ranging from 4 μg/mL to more than 32 μg/mL (Figure 2A). The CLSI breakpoint for high-level vancomycin resistance in enterococci and staphylococci is 32 μg/mL or greater [35]. Because vancomycin resistance in *C. difficile* has not been defined, the nonsusceptible isolates were grouped into 3 categories: (1) low level (≥4–16 μg/mL), (2) intermediate level (16–32 μg/mL), and (3) high level (>32 μg/mL). The different levels of nonsusceptibility observed in the isolates suggest the existence of either different mechanisms or variants of the same mechanism circulating in the patient population. All isolates with vancomycin MIC greater than 32 μg/mL were also resistant to teicoplanin. We also note that vancomycin nonsusceptibility was inducible, as pre-exposure to a subinhibitory concentration resulted in increasing MIC.

The stool samples from Houston were further examined for the year in which they were collected based on vancomycin

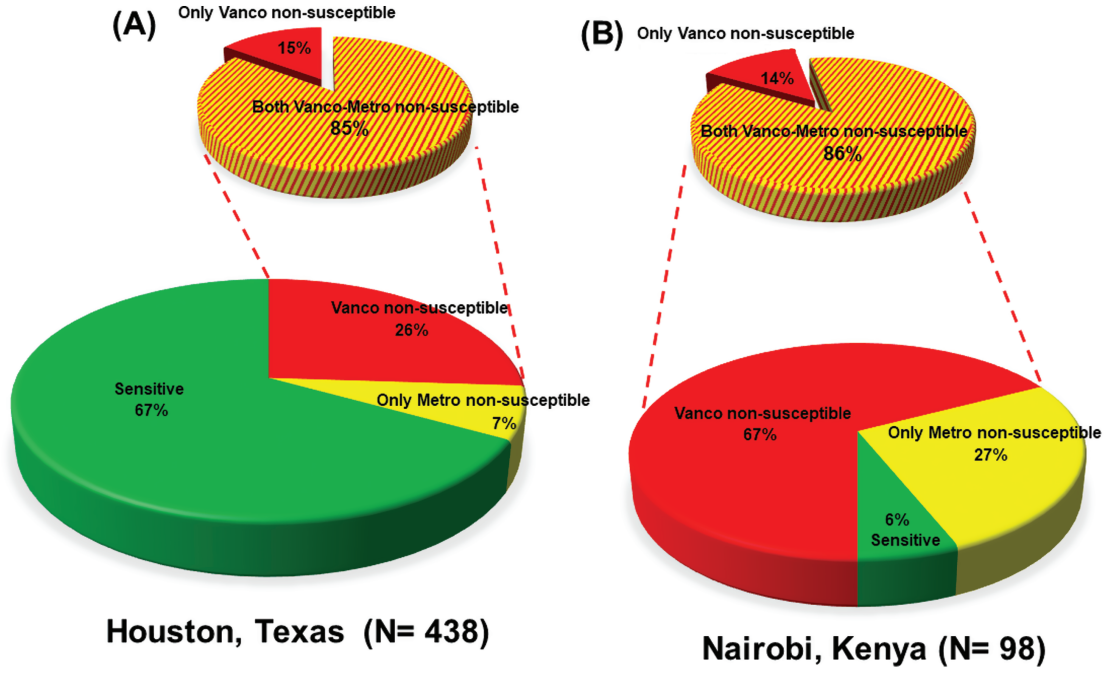

Figure 1. Emergence of metronidazole- and vancomycin nonsusceptible *Clostridioides difficile* isolates in patients from Houston, Texas (*A*), and Nairobi, Kenya (*B*). *Clostridioides difficile*–positive stools from patients with diarrhea were streaked on *C. difficile* medium containing either 8 μg/mL metronidazole or 4 μg/mL vancomycin based on published CLSI break-point concentrations [14–16]. The plates were incubated anaerobically at 37°C for 48 hours and stools that grew colonies were enumerated. Only Metro non-susceptible = stools that only grew metronidazole nonsusceptible isolates; Vanco non-susceptible = stools that grew vancomycin nonsusceptible isolates; Only Vanco non-susceptible = stools that grew only vancomycin nonsusceptible isolates; Both Vanco-Metro non-susceptible = stools that grew both metronidazole and vancomycin nonsusceptible isolates. Abbreviation: CLSI, Clinical and Laboratory Standards Institute.

nonsusceptibility. The results identified an increasing number of stools containing vancomycin nonsusceptible *C. difficile* isolates from 2012–2017 (Figure 2B). The proportion of patients whose stools contained vancomycin nonsusceptible *C. difficile* increased from 2012 to 2017, ranging from complete absence in 2012 to approximately 35% in 2017. However, vancomycin MICs of the isolates remained relatively stable over the years.

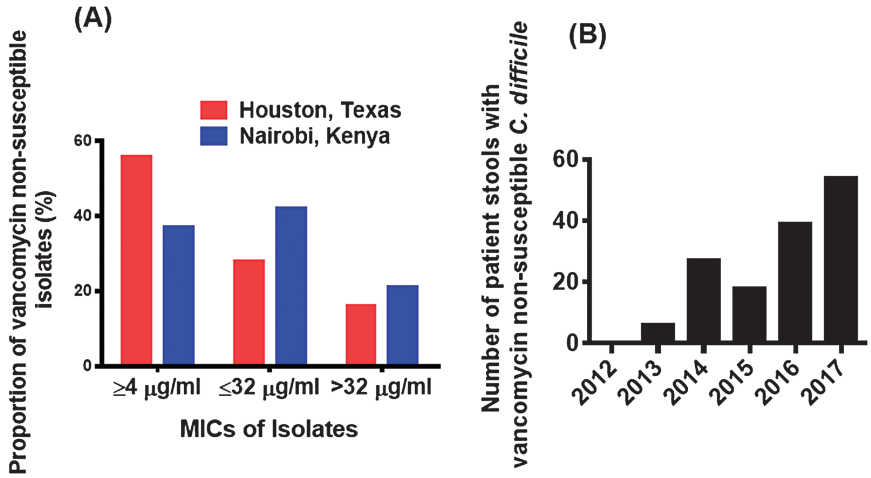

Figure 2. *A*, Distribution of the MICs of vancomycin nonsusceptible *Clostridioides difficile* isolates from patients from Houston, Texas, and Nairobi, Kenya. Vancomycin MIC was determined using Etest (BioMérieux, France) and confirmed with the broth microdilution method [27, 28]. The nonsusceptible isolates were grouped into 3 categories: (2) low level (≥4–16 μg/mL), (2) intermediate level (16–32 μg/mL), and (3) high level (>32 μg/mL). *B*, Number of Houston patients with CDI whose stools contained vancomycin nonsusceptible *C. difficile* isolates from 2012–2017. The number of patients harboring vancomycin nonsusceptible *C. difficile* increased from 2012 to 2017 as follows: 2012 (0), 2013 (1%), 2014 (10%), 2015 (11%), 2016 (22%), and 2017 (35%). Vancomycin CLSI cutoff = 4 μg/mL [14–16]. Abbreviations: CDI, *Clostridioides difficile* infection; CLSI, Clinical and Laboratory Standards Institute; MIC, minimum inhibitory concentration.

Whole-genome sequencing of 10 nonsusceptible isolates did not identify the *vanA* gene cluster, previously associated with high-level vancomycin resistance [7]. Together, these results suggest unknown genetic elements associated with vancomycin nonsusceptibility in isolates circulating in the patient population.

To assess the ability of the vancomycin nonsusceptible isolates to cause disease, mice (both males and females, n = 12 in each group) were either infected with the vancomycin-sensitive R20291 strain or one of the vancomycin nonsusceptible isolates. The infected mice were treated daily with and without vancomycin orally (20 mg/kg) for 4 days and monitored for 14 days to evaluate response to treatment. Only 25% of the mice infected with the vancomycin nonsusceptible isolate survived 5 days postinfection compared with 50% of the mice infected with the vancomycin-sensitive R20291 strain (Figure 3A). Moreover, 75% of mice infected with the R20291 strain and treated with vancomycin survived 14 days postinfection. In contrast, only 30% of the vancomycin-treated mice infected with the vancomycin nonsusceptible isolate survived 5 days postinfection and all of them succumbed by day 10. Gehan-Breslow-Wilcoxon test showed a significant difference (p = .0064) in survival between the vancomycin-treated mice infected with the nonsusceptible isolate and the sensitive strain, but no significant difference (p = .4016) in survival between the untreated mice infected with the nonsusceptible isolate and sensitive strain. Furthermore, the relative amount of toxins A and B present in the pellets of the vancomycin-treated mice infected with the sensitive strain was significantly lower (p = .0025) than in mice infected with the vancomycin nonsusceptible isolate (Figure 3B). Together, these results show that the vancomycin nonsusceptible isolates can cause disease and that therapy with vancomycin is suboptimal to treat the infection.

DISCUSSION

The number of antibiotics currently available for CDI treatment are dwindling because of the intrinsic ability of *C. difficile* to resist multiple drugs [36, 37]. Oral vancomycin is one of the few drugs currently recommended for CDI treatment. Here, we show that *C. difficile* isolates exhibiting reduced susceptibility to vancomycin are emerging in the patient population in 2 distinct geographic locations on different continents. Indeed, our results indicate that 26% and 67% of Houston and Nairobi patients with CDI diarrhea, respectively, harbored *C. difficile* isolates that were nonsusceptible to vancomycin. These nonsusceptibility rates significantly exceeded prior reports from other studies conducted in the United States and Europe from 2011 to 2014,

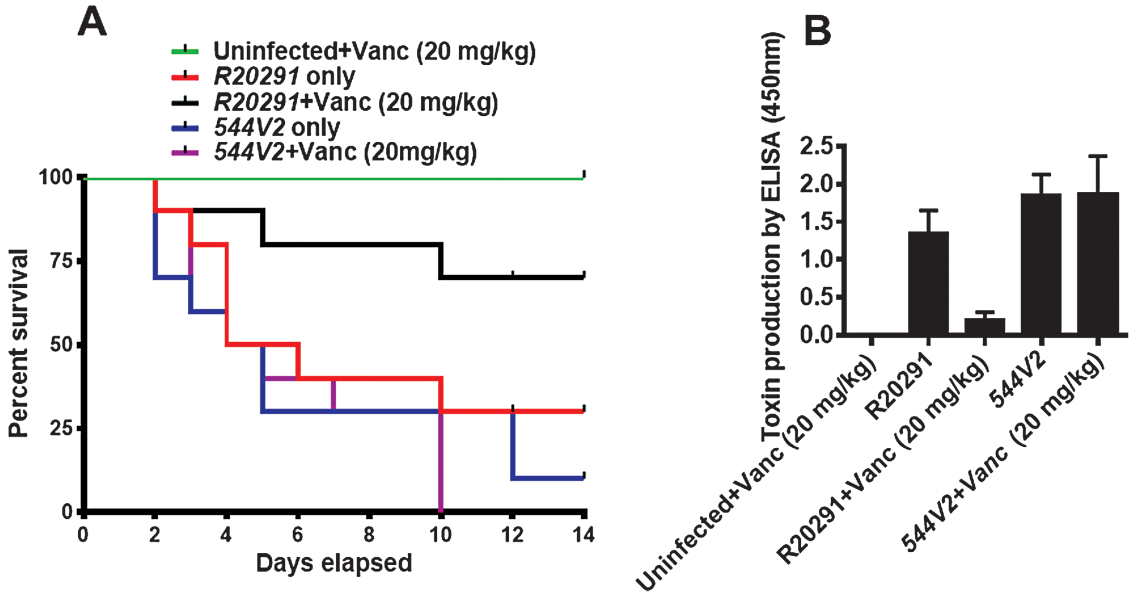

Figure 3. *A*, Vancomycin failed to treat mice infected with a vancomycin nonsusceptible *Clostridioides difficile* isolate in a CDI model. Seven-week-old C57BL/6 mice (both males and females, n = 12 in each group) were first treated with a cocktail of antibiotics and infected with either a vancomycin-sensitive R20291 strain or a vancomycin nonsusceptible isolate (*544V2*). The infected mice were treated with and without vancomycin (20 mg/kg) for 4 days and monitored for 14 days. The mice were scored independently by 2 people based on endpoint symptoms: (1) diarrhea, hunched posture, and physical appearance; (2) movement and response to external stimuli; and (3) bodyweight changes. *B*, Decreased amount of *C. difficile* toxins A and B in the pellets of vancomycin-treated mice infected with the vancomycin-sensitive R20291 strain, but not in treated mice infected with the nonsusceptible isolate, *544V2*. Pellets from the mice collected on day 5 were tested for the presence of toxins A and B using the Wampole *C. difficile* TOX A/B II assay (TechLab, Blacksburg, VA). Gehan-Breslow-Wilcoxon test showed a significant difference (P = .0064) in survival between the vancomycin-treated mice infected with the nonsusceptible isolate *544V2* and the vancomycin-sensitive R20291 strain, but no significant difference (P = .4016) between the untreated mice infected with the nonsusceptible isolate and the sensitive strain. Data presented are the average measurements from the mice in each group. Error bars represent the standard deviation in each group. Abbreviation: CDI, *Clostridioides difficile* infection.

suggesting a lower percentage of resistance to both metronidazole and vancomycin [16]. Our data also suggested a temporal increase in vancomycin nonsusceptibility since 2012 and a greater tendency was observed in Kenya for *C. difficile* isolates to be both metronidazole and vancomycin nonsusceptible.

Metronidazole is poorly bioavailable in the colon, with fecal concentrations ranging from 0.8 to 24.2 μg/g stool [38], possibly explaining metronidazole nonsusceptibility, where exposure to subinhibitory concentrations could have contributed to selection of resistance. On the other hand, fecal concentrations of oral vancomycin are much higher, ranging from 520 to 2200 μg/g stool [39]. Consequently, reduced susceptibility to vancomycin may pose major implications for CDI treatment. Indeed, our *in vivo* data indicated that vancomycin was suboptimal in treating mice infected with a vancomycin nonsusceptible isolate. To date, no functional gene cluster mediating vancomycin resistance has been identified in *C. difficile* [36] and establishing the mechanism of resistance in the vancomycin nonsusceptible *C. difficile* isolates is the focus of our ongoing work.

Our results may help explain a decreasing effectiveness of antibiotic-based therapy in CDI since a significant proportion of patients harboring strains with reduced susceptibility to vancomycin may not respond to treatment. Studies of susceptibility will be needed for other drugs, including fidaxomicin, tigecycline, ramoplanin, rifaximin, and nitazoxanide, to help guide recommendations for future therapy. The spread of *C. difficile* strains that are not susceptible to vancomycin will produce challenges to therapy for this common infection and has serious public health implications, underscoring an urgent need for a comprehensive analysis of the circulating strains to help inform clinical decisions. Most importantly, routine susceptibility testing, specific definitions for resistance, and molecular detection of genes involved in the mechanism of vancomycin nonsusceptibility in pathogenic *C. difficile* strains will need to be expanded.

Notes

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