Infection of Hamsters with Historical and Epidemic BI Types of Clostridium difficile

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Background. North American and European hospitals have reported outbreaks of Clostridium difficile–associated disease with unexpectedly high mortality caused by a newly recognized group of C. difficile strains, group BI. Our objective was to compare, in hamsters, the virulence of a historical nonepidemic BI type, BI1, with that of 2 recent epidemic BI types, BI6 and BI17, and with that of 2 standard toxigenic strains, K14 and 630.

Methods. For each strain, 10 hamsters were given 1 dose of clindamycin, followed 5 days later with 100 C. difficile spores administered by gastric inoculation. Outcomes were recorded.

Results. The hamster model demonstrated variations in mean times from inoculation to death (for BI6, 40 h; for BI1, 48 h; for K14, 49 h; for BI17, 69 h; for 630, 102 h; for BI6, BI1, and K14 vs. 630, P < .01; for BI17 vs. 630, P < .05) and from colonization to death (for BI1, 7 h; for BI17, 13 h; for BI6, 16 h; for K14, 17 h; for 630, 52 h; for BI1, BI17, BI6, and K14 vs. 630, P < .01).

Conclusion. Group BI strains were not more rapidly fatal than the standard toxinotype 0 strain K14 but were more rapidly fatal than the standard toxinotype 0 strain 630. BI6, the most common BI type in our collection, was particularly virulent in hamsters, consistently causing death within 48 h of inoculation.
Table 1. Characteristics of Clostridium difficile strains studied.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Toxinotype</th>
<th>Epidemic or nonepidemic</th>
<th>Source</th>
<th>Clindamycin susceptibility</th>
<th>Fluoroquinolone susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>K14</td>
<td>5780</td>
<td>Epidemic</td>
<td>Chicago, 1994</td>
<td>Resistant</td>
<td>Susceptible</td>
</tr>
<tr>
<td>R23</td>
<td>630b</td>
<td>Nonepidemic</td>
<td>Switzerland, 1980s</td>
<td>Resistant</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Bi1</td>
<td>1675</td>
<td>Nonepidemic</td>
<td>Minneapolis, 1988</td>
<td>Susceptible</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Bi6</td>
<td>6336</td>
<td>Epidemic</td>
<td>Portland, Maine, 2003</td>
<td>Resistant</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Bi17</td>
<td>6443</td>
<td>Epidemic</td>
<td>Montreal, 2004</td>
<td>Susceptible</td>
<td>Susceptible</td>
</tr>
</tbody>
</table>

NOTE. All isolates used in this study were from cases of human disease. REA, restriction endonuclease analysis.

a Fluoroquinolone susceptibility refers to gatifloxacin and moxifloxacin. Clinical and Laboratory Standards Institute breakpoints for trovafloxacin were used in the absence of set breakpoints for C. difficile. Susceptibility breakpoints were as follows: for fluoroquinolones, 2 μg/mL for susceptible and 8 μg/mL for resistant; for clindamycin, 3 μg/mL for susceptible and 8 μg/mL for resistant. Isolates used in this study were resistant to clindamycin (≥256 μg/mL) [15]. All isolates were resistant to ciprofloxacin.

b We refer to this isolate (REA type R23) as strain 630 in the text, in keeping with previous designations used in the literature [16].

Type 3 strains, which produce binary toxin and contain the tcdC gene deletion. However, no epidemics have been associated with the previously uncommon historical BI types, designated BI1 through BI5 on the basis of their closely related but distinct REA patterns. Furthermore, historical BI types were susceptible to newer fluoroquinolones, whereas the recent outbreak-associated BI types have demonstrated resistance to the fluoroquinolones gatifloxacin and moxifloxacin [7, 8].

The Syrian golden hamster model of CDAD parallels most of the important aspects of human infection, particularly regarding susceptibility to C. difficile infection after antimicrobial administration [12]. After clindamycin administration and challenge with toxigenic C. difficile strains, hamsters develop a hemorrhagic cecitis, which manifests as “wet tail” (a sign of diarrhea in hamsters) and is followed by death. Hamsters frequently die of the infection precipitously (often without signs of wet tail) when infected with highly virulent C. difficile strains. In the present study, we followed a previously published protocol for C. difficile infection, using a low inoculum of spores, the presumed infectious form of C. difficile, as most likely occurs in the hospital setting [13]. Following this previously published protocol allowed us to make comparisons between results for other C. difficile strains and the current epidemic BI types.

The purpose of the present study in the hamster CDAD model was 2-fold: (1) to compare the time to death for BI group strains with that for 2 standard (toxinotype 0) toxigenic strains of C. difficile (K14, an epidemic strain previously studied in the same protocol, and 630, a nonepidemic strain for which the genome has been fully sequenced) and (2) to compare the time to death for a historical nonepidemic BI type, BI1, with that for 2 recent distinct epidemic BI types, BI6 and BI17.

MATERIALS AND METHODS

Spore preparation. C. difficile was inoculated anaerobically on anaerobic blood agar plates and incubated at 37°C until colonies were confluent. The plates were maintained for 3 days to induce sporulation. The organisms were harvested with disposable loops, placed into 10 mL of PBS with no added calcium or magnesium, washed in PBS, and heat-shocked at 56°C for 10 min to kill surviving vegetative cells. The spores were centrifuged and resuspended in Dulbecco’s modified Eagle medium (DMEM), aliquoted, and frozen at −80°C. The frozen spores were quantitated before use by plating 100 μL of 10-fold serial dilutions of the spores onto taurocholate fructose agar plates [14]. Spores were diluted in DMEM for orogastric inoculation into hamsters; 1 μL of green food coloring was added to the inoculum for ease of visibility, to ensure that hamsters received the entire dose.

C. difficile strains. C. difficile strains were selected on the basis of epidemiologic and toxigenic characteristics, as shown in table 1. Each strain can be categorized as standard (toxinotype 0) or variant (toxinotype 3). Toxinotype 0 strains produce an enterotoxin, toxin A, and a cytotoxin, toxin B. Toxinotype 0 strains included REA type K14, the most common strain recovered from 2 Chicago hospitals with sustained epidemic or high endemic rates of CDAD in 1994 and 1995 [17], and strain 630, a nonepidemic strain that was first isolated in Switzerland and that is the first C. difficile strain for which the genome was fully sequenced.

Toxinotype 3 strains also produce toxins A and B but are characterized by specific genetic restriction fragment–length polymorphisms within the pathogenicity locus of C. difficile in which the genes for toxin A and B are located [9]. The toxinotype 3, REA group BI strains produce an additional toxin, binary toxin, and have an 18-bp deletion in the tcdC gene, a putative down-regulator of toxins A and B. Toxinotype 3 strains included BI1, a historical nonepidemic BI type with susceptibility to fluoroquinolones, and BI6 and BI17, recent epidemic BI types with resistance to fluoroquinolones. BI1 is 1 of 5 historical BI types identified in a database of >6000 C. difficile samples maintained by Hines Veterans Affairs (VA) investigators. The database includes 5 BI1 isolates collected from 4 different patients. The BI1 isolate used in this study (1675) dates from 1988 (Minneapolis, Minnesota). BI6 (6336), a recent epidemic isolate, was part of a CDAD outbreak in a Portland, Maine, hospital in 2003. BI17 (6443), a
recent epidemic isolate, was part of a CDAD outbreak in a Montreal, Quebec, hospital in 2004.

Clindamycin and fluoroquinolone susceptibilities were determined by use of a modified E-test, using Clinical and Laboratory Standards Institute (CLSI) breakpoints for trovafloxacin to assess gatifloxacin and moxifloxacin [13, 15]. Clindamycin was included because the study design used clindamycin to cause hamsters to be susceptible to \textit{C. difficile} infection. It was postulated that isolate susceptibility might decrease the percentage of hamsters colonized or increase the time from inoculation to colonization.

**Animal studies.** Experimental protocols were approved by the Institutional Animal Care and Use Committee and the Research and Development Committee of the Hines VA Hospital research service. Adult (6 weeks at delivery) male Syrian golden hamsters (90–100 g) were obtained from Charles River Laboratories. Hamsters were housed individually in policarbonate cages fitted with filter covers that hold disposable polyester air filters. All food, water, bedding, cages, wire lids, and filter covers were autoclaved before use. Each group of 10 hamsters was housed in a separate room to prevent cross-contamination with different strains of \textit{C. difficile}. The standardized published protocol for clindamycin was followed [13]. The K14, 630, B11, B16, and B17 isolates were each assayed in groups of 10 hamsters. For each isolate, hamsters were given 1 dose of clindamycin orogastrically (30 mg/kg) on day 0, to establish susceptibility to \textit{C. difficile} infection. This was followed on day 5 by gastric inoculation with 100 colony-forming units of the designated \textit{C. difficile} spores. Immediately preceding treatment with clindamycin, bedding was changed, and fecal pellets were collected for culture on medium selective for \textit{C. difficile}. This confirmed that hamsters were not colonized with \textit{C. difficile} before the administration of clindamycin. Hamsters were monitored at 8-h intervals for signs of \textit{C. difficile} infection. Signs of infection, including stiffness, lying prone, wet tail, diarrhea, and death, were monitored and recorded. Hamsters found lying prone or unresponsive were euthanized.

Experience with this hamster model of CDAD has indicated that standard toxigenic epidemic strains of \textit{C. difficile} cause death at ~48 h. In the previous study [13], hamsters had been monitored at 12-h intervals, but, to detect a difference in time to death between standard and variant toxigenic isolates, monitoring frequency was increased to 8-h intervals in the present study.

Fecal pellets were collected daily, including on the day of death, and were cultured anaerobically on selective TCCFA (taurocholate–cycloserine–cefoxitin–fructose agar) plates to determine time to colonization. For hamsters that were not colonized, fecal pellets were collected daily for 12 days and then were collected weekly until termination of the study. Bedding was changed daily. \textit{C. difficile} from at least 3 colonized hamsters per group was typed by REA to confirm the identity of the infecting \textit{C. difficile} isolate. Study groups were assessed for percentage of hamsters colonized, time between challenge with \textit{C. difficile} and colonization, time between challenge with \textit{C. difficile} and death, time between colonization with \textit{C. difficile} and death, and morbidity before death.

**REA typing.** \textit{C. difficile} isolates were inoculated into a trypticase soy broth from a 48-h culture on blood agar plates and were incubated anaerobically overnight. The DNA was isolated as described elsewhere [10] and then was digested with \textit{Hind}III. The fragments were separated on a 0.7% agarose gel, producing a characteristic banding pattern for each isolate. The REA typing system identifies a closely related group (differing by ~6 bands) by letter(s) and specific types (identical banding patterns) within that group by number.

**Measurement of toxin production in vitro.** Pure \textit{C. difficile} strains were inoculated from freezer cultures onto anaerobic blood agar plates and incubated for 48 h at 36°C, after which single colonies were inoculated into separate tubes containing 20 mL of prereduced brain-heart infusion (BHI) broth (Difco BHI; DF0037-17-8), which were placed in the anaerobic incubator for 24 and 72 h (1 tube for each time point). Previous incubation in BHI broth had demonstrated equivalent growth curves (data not shown). At each time point, 3 mL of supernatant was withdrawn from the broth culture and centrifuged at 2500 g for 10 min; 1.5 mL of supernatant was transferred to a sterile tube and then recentrifuged in a microfuge at 16,000 g for 2 min, and 1 mL of supernatant was stored at 4°C until testing. Toxin standards were made from purified \textit{C. difficile} toxin A (gift from D. Lyerly, TechLab). The purified toxin A was diluted in sterile dH2O (molecular biology reagent grade; Sigma; W4502) to final concentrations of 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 ng/mL; 100 µL of each toxin standard or unknown supernatant was added to separate wells containing \textit{C. difficile} toxin antibody conjugate (TechLab A/B Kit). The assay was done per the kit’s instructions. The plate was read on a Bio-Rad ELISA plate reader at 450 nm, and the toxin standard values were plotted on a curve, which was linear from 0.5 to 20 ng/mL. Supernatant toxin concentrations were extrapolated from the optical-density values within the range of the linear standard curve by doing 10-fold dilutions.

**Statistical methods.** Percentage of hamsters colonized, mean time between challenge with \textit{C. difficile} and colonization, mean time between challenge with \textit{C. difficile} and death, and mean time between colonization with \textit{C. difficile} and death were compared using 1-way analysis of variance. For the time between colonization with \textit{C. difficile} and death, time to colonization was recorded in hours, because, even though colonization was assessed daily, death was assessed every 8 h. For hamsters found dead before daily colonization was confirmed, colonization was presumed to have occurred on the same day as death, and the time between colonization and death was considered to be 0 h. Kaplan-Meier survival curves were calculated to depict survival after challenge and after colonization, by use of Stata/SE (version...
RESULTS

Characteristics of the tested organisms are shown in table 1. The BI1 and BI17 isolates were susceptible to clindamycin, whereas other isolates were resistant to clindamycin (MIC > 256 μg/mL). BI6 and BI17 were resistant to gatifloxacin and moxifloxacin by use of the CLSI breakpoint for trovafloxacin of 8 μg/mL. The other isolates were susceptible. All isolates were resistant to ciprofloxacin.

The percentage of hamsters colonized differed among the groups, but the difference was not statistically significant. Strain 630 colonized 100% of hamsters inoculated (10/10). K14, BI1, and BI6 colonized 90% of hamsters (9/10), and BI17 colonized 80% (8/10). Colonized hamsters demonstrated 100% mortality. Mean times from challenge with C. difficile on day 5 to colonization differed among the strains (figure 1). No significant difference in time to colonization was detected between the standard and variant toxigenic strains. No significant difference was detected between the historical type BI1 and recent epidemic BI types; however, the recent epidemic type BI6 colonized hamsters more rapidly than did the recent epidemic type BI17 (P < .05).

The hamster model demonstrated variations in the mean time between challenge with C. difficile on day 5 and time to death (figure 2). BI6 caused death in hamsters most rapidly at a mean ± SE of 40 ± 2.1 h, followed by BI1 at 48 ± 3.5 h, K14 at 49 ± 3.4 h, BI17 at 69 ± 14.7 h, and 630 at 102 ± 7.8 h (for BI6, BI1, and K14 vs. 630, P < .01; for BI17 vs. 630, P < .05). Other differences did not reach significance.

The hamster model also demonstrated variations in the mean time between colonization with C. difficile and death (figure 3). BI1 caused death in hamsters most rapidly after colonization at a mean ± SE of 7 ± 2.1 h, followed by BI17 at 13 ± 3.4 h, BI6 at 16 ± 2.1 h, K14 at 17 ± 3.6 h, and 630 at 52 ± 7.8 h (for BI1, BI17, BI6, and K14 vs. 630, P < .01). When the standard and variant toxigenic strains were compared, all 3 toxin-variant BI types were found to cause death more rapidly after colonization than the standard toxigenic strains K14 and 630, but the difference was statistically significant only for 630. Notably, BI17 required more time to colonize than did BI6, but once colonized, these 2 BI types were essentially the same in terms of time to death (figures 1 and 3).

Signs of morbidity (inactivity or recumbency) and wet tail or diarrhea were not always apparent before death (table 2). More hamsters infected with epidemic BI types died without showing any signs of illness than did those infected with 630 and K14, but the difference was not significant. The signs of wet tail or diarrhea were specifically more common in hamsters infected with strain 630 before death (for 630 vs. K14 and BI17, P < .01).

In vitro toxin production is illustrated in figure 4. Strain 630 showed lower toxin production at 24 h (5.5 ng/mL) than did the other strains (for K14, 650 ng/mL; for BI1, 450 ng/mL; for BI6, 400 ng/mL; for BI17, 580 ng/mL). The same trend was found at 9.2; Stata). P < .05 was considered to indicate statistical significance.
72 h (for 630, 650 ng/mL; for K14, 8000 ng/mL; for BI1, 4200 ng/mL; for BI6, 5500 ng/mL; for BI17, 5500 ng/mL).

**DISCUSSION**

In a previous study using this protocol, 5 different toxigenic strains of *C. difficile* of known epidemiologic importance were tested for virulence in hamsters [13]. The previous study demonstrated differences in pathogenicity between toxin-variant and standard toxigenic strains but no significant differences among the standard strains. In contrast, the present study found differences between both toxin-variant and standard toxigenic strains and significant differences among the standard toxigenic strains and among the variant toxigenic isolates.

The epidemic strain of *C. difficile* identified by REA typing as BI has caused recent outbreaks within and outside of the United States, and these outbreaks have caused higher-than-expected mortality [8]. We have used a standardized hamster model to determine whether we can confirm higher morbidity and mortality with toxin-variant strains than with standard toxigenotype 0 strains. We used 3 type BI isolates, 1 historical nonepidemic type (BI1), and 2 recent epidemic types (BI6 and BI17) and compared them with 2 standard toxigenic strains, an epidemic isolate (K14) previously tested in this model, and a nonepidemic strain (630) that has been genetically sequenced. The BI types and K14 caused death more rapidly than did 630, but the BI types tested in this model did not demonstrate a more-rapid time to death than did the standard toxigenic epidemic strain K14.

One possible explanation for the similarity between the K14 and BI isolates in the hamster model is high levels of toxin production, as reported by Warny et al. [6]. In experiments using supernatants from cultures of these isolates, we confirmed that strains that demonstrated a decreased time from challenge to death or a decreased time from colonization to death in the hamster model (K14, BI1, BI6, and BI17) also produced more toxin in vitro than did strain 630, at both 24 and 72 h. However, previous data from hamsters have not always shown a correlation between toxin production in vitro and virulence in the animal model [18].

We did not observe significant differences in time from challenge with *C. difficile* to death or from colonization to death between recent and historical BI types, although the trend was

**Table 2. Signs of morbidity in hamsters before death.**

<table>
<thead>
<tr>
<th>REA type/strain designation</th>
<th>No.</th>
<th>Hours of morbidity before death, range</th>
<th>Any morbidity before death, %</th>
<th>Wet tail (diarrhea) at death, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>K14</td>
<td>9</td>
<td>0–24</td>
<td>88</td>
<td>0%</td>
</tr>
<tr>
<td>630</td>
<td>10</td>
<td>9–32</td>
<td>100</td>
<td>80%</td>
</tr>
<tr>
<td>BI1</td>
<td>9</td>
<td>0–24</td>
<td>67</td>
<td>44%</td>
</tr>
<tr>
<td>BI6</td>
<td>9</td>
<td>0–16</td>
<td>67</td>
<td>44%</td>
</tr>
<tr>
<td>BI17</td>
<td>8</td>
<td>0–16</td>
<td>50</td>
<td>0%</td>
</tr>
</tbody>
</table>

**NOTE.** REA, restriction endonuclease analysis.

* a Signs of stiffness in movement, hunched posture, prone posture, unresponsiveness, and rapid breathing.

* b P < .01 for K14 and BI17 vs. 630.
toxin variant epidemic strains with respect to time to death. A hamster model, an epidemic standard toxigenic strain such as high virulence in humans, but we also demonstrated that, in the morbidity and mortality that are consistent with observations of this hamster model. We were able to observe differences in strain man epidemic strains of may not have been large enough to show subtle differences. Hu-
showing 90% colonization, and 1 showing 100% colonization)ulated per group, with 1 isolate showing 80% colonization, 3 number of hamsters, the number colonized (10 hamsters inoc-
hamster model chosen. Although we used a reasonably large animals [18].

classification as a highly virulent strain [18]. In the present study, the recent epidemic type BI6 was the only strain to fit the crite-
100% mortality within 48 h of inoculation as the criterion for

toward a more-rapid time to death after challenge for 1 of the new epidemic types, BI6. Once colonized, all 3 BI types demon-
strated more-rapid time to death than did the standard toxigenic strain 630 but not K14.

Susceptibility of an isolate to clindamycin could potentially decrease the percentage of hamsters colonized or increase the time from challenge with C. difficile to colonization, because of residual clindamycin in the hamster gut. The clindamycin-susceptible isolate BI1 colonized 90% of hamsters and the clindamycin-susceptible isolate BI17 colonized 80%, but this difference was not statistically significant compared with the other isolates tested. The increased time to colonization for the clindamycin-susceptible types BI1 and BI17 relative to the new clindamycin-resistant epidemic type BI6 (figure 1) may be attributable to this factor, but this difference was statistically significant only for BI6 versus BI17. The clindamycin-resistant isolates 630 and K14 had times to colonization similar to that for the clindamycin-susceptible isolate BI1, indicating that factors other than sus-
ceptibility to clindamycin may also influence time to coloni-
zation. Because the BI types are closely genetically related, the differences observed in time to colonization between types within this REA group are more likely related to clindamycin susceptibility.

A previous study of C. difficile in the hamster model classified toxigenic strains as being highly virulent or less virulent, with 100% mortality within 48 h of inoculation as the criterion for classification as a highly virulent strain [18]. In the present study, the recent epidemic type BI6 was the only strain to fit the crite-
rion for a highly virulent strain—all 9 colonized hamsters died within 48 h of inoculation. In contrast, isolate 630 responded significantly differently from the 4 other strains in the present study, demonstrating a delay in time from challenge with C. difficile to death and from colonization to death. Strain 630 met the criterion for being a lower virulence organism in the hamster model, with death occurring 5 days after inoculation in some animals [18].

A potential limitation of the present study is the very sensitive hamster model chosen. Although we used a reasonably large number of hamsters, the number colonized (10 hamsters inoculated per group, with 1 isolate showing 80% colonization, 3 showing 90% colonization, and 1 showing 100% colonization) may not have been large enough to show subtle differences. Hu-
man epidemic strains of C. difficile predictably caused disease in this hamster model. We were able to observe differences in strain morbidity and mortality that are consistent with observations of high virulence in humans, but we also demonstrated that, in the hamster model, an epidemic standard toxigenic strain such as K14 may be statistically indistinguishable from the more recent toxin variant epidemic strains with respect to time to death. A prior study [13] of epidemic toxinotype 0 strains (namely, K14, J9, and B1) in this model showed results similar to those found for K14, BI1, and BI6 in the present study. Perhaps the most compelling data from the present study is the rapidity of death after colonization by the recent and historical epidemic BI types and the high number of deaths in hamsters with no prior signs of morbidity. Both of these observations could be indicative of epidemic strains that are capable of causing more-rapid disease and more-rapid death, consistent with the in vivo observations in patients and the in vitro evidence of increased toxin production in these strains [6].

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