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In vitro assessment of *Clostridium difficile* PCR ribotype 002: the most prevalent *C. difficile* ribotype in the United Kingdom.

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Abstract (amended)

Background: *Clostridium difficile* infection (CDI) causes substantial morbidity and healthcare expenditure across Europe. UK prevalence of *C. difficile* PCR ribotype 027 (NAP1) has declined dramatically recently and other ribotypes have emerged, including ribotype 002 (CD002); now the most prevalent UK ribotype. CD002 is also responsible for CDI in many countries across Europe, including: France, Germany, Ireland, and The Netherlands. We assessed the *in vitro* phenotypic characteristics of CD002 from across Europe to determine traits that may contribute to its increasing clinical prevalence.

Material/methods: Sixty CD002 were studied: UK isolates from 2007-2008 (geographically distinct, N=15), UK isolates from 2011-2013 (19 locations, N=22), and non-UK European isolates from 2012-2014 (N=23, 20 locations). Antimicrobial susceptibilities (13 antimicrobials) were evaluated using an agar incorporation method. Maximum specific growth rates (μmax) were calculated and cytotoxin titres (log_{10}-relative units, RU) determined using Vero cell cytotoxicity assays. Biofilm formation was quantified using 96-well microtitre plate assays and sporulation capacities assessed in liquid culture by quantifying spore-formation over 120 h (CFU/mL).

Results: All isolates were susceptible metronidazole, vancomycin, tetracycline and linezolid (MICs ≤2 mg/L). Clindamycin resistance (MIC ≥8 mg/L) was more common in non-UK CD002 (30%) than UK strains (5-13%). Resistance to erythromycin, clarithromycin, nitrofurantoin, chloramphenicol, and moxifloxacin was uncommon (5-7%). MICs for penicillin's remained below resistance breakpoints, regardless of origin, in all but one isolate (ampicillin MIC 2 mg/L). All CD002 were resistant to trimethoprim (MICs >128 mg/L) and ciprofloxacin (MICs ≥8 mg/L). One MDR strain (UK, 2007) was observed that was macrolide, fluoroquinolone, ampicillin, and nitrofurantoin resistant. Significantly faster μ_{max} was seen in non-UK CD002 (0.92 ±0.058 h^{-1}) than recent/older UK strains (0.76 ±0.063/0.69 ±0.028 h^{-1} respectively) (P<0.001). Cytotoxin production did not differ significantly (median titres 2-3 RU) between CD002 groups. Recent UK/non-UK CD002 formed significantly greater biofilms by 3 days than asynchronous UK CD002 (P<0.001). Sporulation studies demonstrated that recent UK/non-UK CD002 sporulated more at 24 h than older UK CD002; 18.6-fold/31.2-fold respectively (P<0.05), but by 120 h sporulation did not differ.

Conclusions: Recent CD002 from diverse European locations were assessed for traits that may help to explain emergence of CD002 in the UK and compared to asynchronous CD002. Previous studies demonstrated elevated CD002 μ_{max} compared to hypervirulent ribotypes 027/078; and the present study demonstrated that recent non-UK CD002 μ_{max} were significantly further elevated vs. UK isolates. Non-UK CD002 were more clindamycin resistant, but other antimicrobial susceptibilities were similar between CD002 groups. Recent CD002 demonstrated significantly increased sporulation capacities at 24 h and more extensive 3 day biofilm formation compared to asynchronous UK CD002, which could enhance their survival and transmission early in an episode CDI. Further phenotypic and genetic studies are required to evaluate further characteristics of CD002 that may be associated with its emergence in the UK.

Introduction

- Despite improved clinical management strategies for CDI, healthcare costs for treating CDI remain high and have been estimated in the USA at $1.1-3.2 billion [1-2].
- *C. difficile* hypervirulence has been attributed to ribotypes 027 & 078 due to increased CDI severity [3-4].
- UK: national distribution of *C. difficile* ribotypes is monitored by the *C. difficile* Ribotyping Network and CD002 is now the most common ribotype in the UK, with prevalence in 2015 of 14.5% (Q1), 18.4% (Q2), 15.0% (Q3), and 16.4% (Q4) of isolates submitted to CDRN [5]
- Europe: CD002 has been isolated in: France, Germany, Ireland, and The Netherlands.

Materials & Methods

***C. difficile* strains (Figure 1)**

- 60 CD002 : UK isolates, 2007-2008 (geographically distinct, N=15); UK isolates, 2011-2013 (19 locations, N=22); and non-UK European isolates, 2012-2014 (N=23, 20 locations).

Antimicrobial susceptibility testing

- Agar incorporation MICs on Wilkins-Chalgren agar with 10^4 *C. difficile* cfu per spot

Biofilm quantification

- Culture medium: BHI+0.1% (w/v) L-cysteine HCl + 0.5% (w/v) yeast extract + 0.1M glucose (BHISG). Quantification: 3 and 6 days, crystal violet staining (OD_{590})

Growth rate analysis & cytotoxin production

- Growth rate analysis: OD_{600} determined at time between 3-6 hours during log growth
- Cytotoxin: Vero cell cytotoxicity assay of BHIS culture supernatants from 72 h cultures
- Cytotoxin titres expressed as log_{10} relative units (RU)

Results

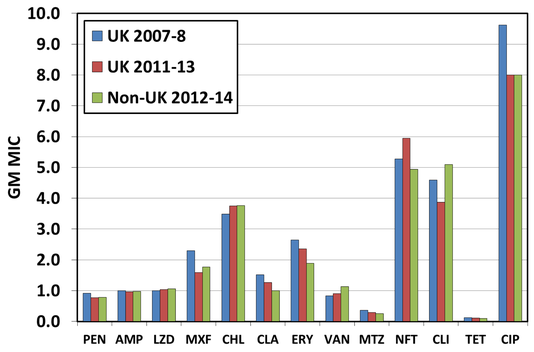

Figure 2. Antimicrobial agent geometric mean (GM) MICs (mg/L) for CD002 using agar incorporation MIC testing. PEN, penicillin G; AMP ampicillin; LZD, linezolid; MXF, moxifloxacin; CHL, chloramphenicol; CLA, clarithromycin; ERY, erythromycin; VAN, vancomycin; MTZ, metronidazole; NFT, nitrofurantoin; CLI, clindamycin; TET, tetracycline; CIP, ciprofloxacin. Differences between groups are non-significant.

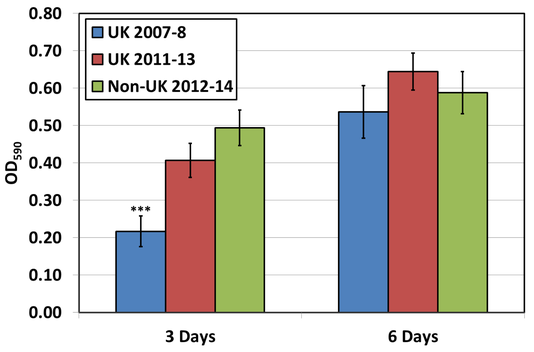

Figure 3. *C. difficile* ribotype 002 mean (±SE) biofilm production (OD_{590}) in a microtitre plate assay with crystal violet staining after 3 and 6 days anaerobic incubation in BHISG. ***significant difference P<0.001.

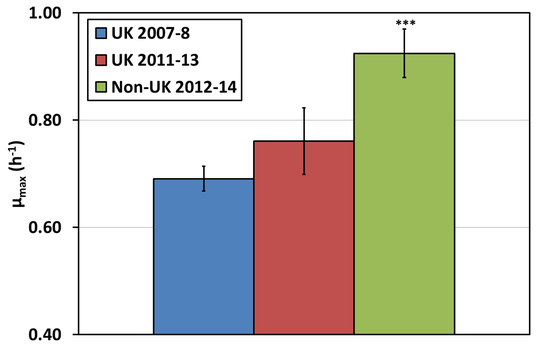

Figure 4. Maximum specific growth rates (μ_{max}) of CD002 (h^{-1}, mean ±SE) in BHIS during exponential growth. ***significant difference P<0.001.

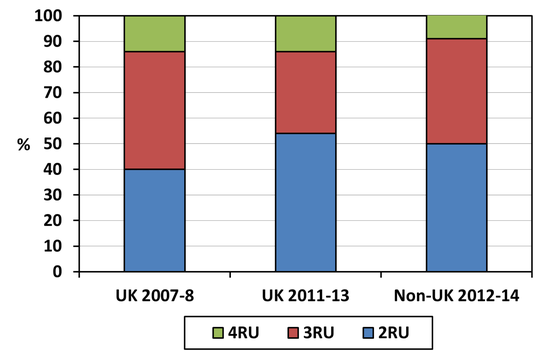

Figure 5. Percentage (%) of CD002 isolates demonstrating cytotoxin titres of 2 RU, 3 RU, and 4RU after 72 hours following culture in BHIS. Differences between groups are non-significant.

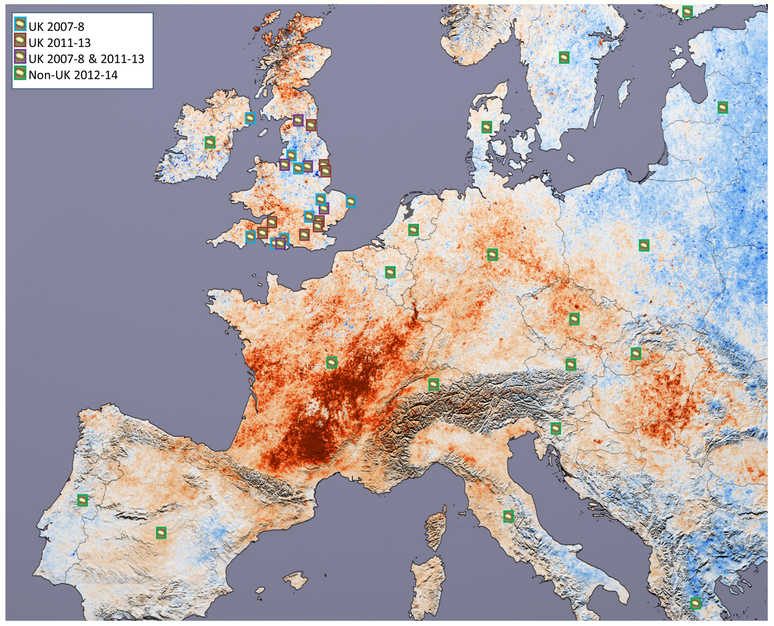

Figure 1. Distribution of CD002 isolates evaluated in this study. Non-UK CD002 makers are correct to country but not exact geographical location (Contour coloration is not relevant to this study map acquired from www.nasa.gov)

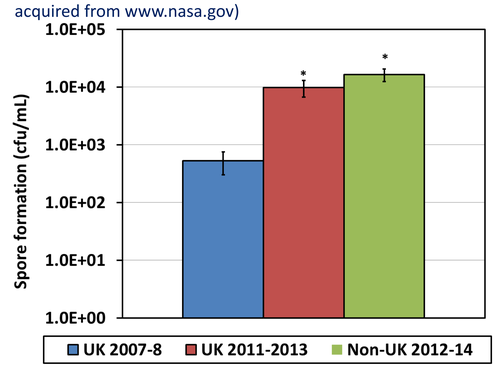

Figure 6. Spore formation (cfu/mL) by CD002 in BHIS broth after 24 hours incubation.

Results/Conclusions

- Antimicrobial susceptibilities (Figure 2) did not differ significantly between CD002 from different locations or lineages; therefore, these are unlikely to be a driver for the emergence of CD002 in the UK.
- Biofilm formation was significantly enhanced in more recent CD002 isolates(Figure 3, P<0.001) after 3 days, although not significantly greater after 6 days.
- Non-UK CD002 grew significantly (P<0.001) faster than UK strains (Figure 4), regardless of year of isolation.
- All CD002 had elevated growth rates compared with hypervirulent ribotypes 027 & 078 (data not shown), which could confer a competitive advantage.
- More recent CD002 sporulated to a significantly greater degree after 24 hours compared with older UK isolates (Figure 6, P<0.05) and toxin titres were comparable with those for hypervirulent ribotypes (data not shown).
- Enhanced CD002 sporulation, as observed in prior studies [6], & elevated biofilm formation may benefit recent isolates and provide a competitive advantage *in vivo;* studies of the kinetics of toxin production are warranted.

References

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