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

Ameh, IL\(^1\); Freeman, J\(^2\); Fawley, WN\(^3\); Wilcox, MH\(^2,3\); Baines, SD\(^1\).

\(^1\)Clostridium difficile* Research Laboratory, Department of Biological & Environmental Sciences, Univ. Hertfordshire, Hatfield, AL10 9AB, UK. s.baines2@herts.ac.uk.

\(^2\)Leeds Teaching Hospitals NHS Trust, Old Medical School, Leeds General Infirmary, Leeds LS1 3EX, UK.

\(^3\)CDRN Public Health England, Old Medical School, Leeds General Infirmary, Leeds LS1 3EX, UK.

Abstract (amended)

*Background:* *Clostridium difficile* infection (CDI) causes substantial morbidity and healthcare expenditure across Europe. UK prevalence of *C. difficile* PCR ribotype 002 (NDAP1) has increased 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 expressed as 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%). 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 (MIC >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\(_{\text{max}}\) 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\(_{\text{max}}\) compared to hypervirulent ribotypes 027/078; and the present study demonstrated that recent non-UK CD002 μ\(_{\text{max}}\) 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 epidemic 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)
  - Antimicrobial susceptibility testing
    - *Agar* incorporation MICs on Wilkins-Chalgren agar with 10\(^{-6}\) * C. difficile cfu per spot
  - *Culture* media: BH:Ho+0.1% (w/v) L-cysteine HCl + 0.5% (w/v) yeast extract + 0.1% glucose (BHI).
  - Quantification: 3 and 6 days, crystal violet staining (OD\(_{600}\))
  - *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 BHS culture supernatants from 72 hours cultures
    - *Cytotoxin* titres expressed as log\(_{10}\) relative units (RU)

References