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Phenotypic evaluation of *Clostridium difficile* PCR ribotypes 078, 027, and 002: antimicrobial susceptibilities, cytotoxin production profiles, and biofilm formation.

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Abstract (amended)

Objectives: Specific *Clostridium difficile* ribotypes have been designated as hypervirulent, e.g. PCR ribotypes 027 (NAP1/BI) and 078 (NAP7/BK), principally based upon clinical observations of *C. difficile* infection (CDI) severity. Although early ribotype 027 studies highlighted potential elevated toxin production *in vitro*, subsequent research has not corroborated these findings when comparing with other prevalent *C. difficile* ribotypes. We assessed the *in vitro* phenotypic characteristics of three common ribotypes (002, 027, and 078), collected from diverse sources, to determine potential traits that may contribute to their high clinical prevalence.

Methods: Three ribotype groups of *C. difficile* isolates were obtained from 13 NHS hospitals: 002 (n=11, 6 locations), 027 (n=11, 5 locations), and 078 (n=11, 5 locations), plus ATCC 700057 and internal control (E4, ribotype 010) control strains. Antimicrobial susceptibilities to: linezolid, ciprofloxacin, moxifloxacin, metronidazole, vancomycin, erythromycin, tetracycline, and clindamycin were determined using an agar incorporation method. Growth kinetics were assessed in BHIS broth, maximum specific growth rates (μ_{max}) calculated, and cytotoxin titres (log_{10}-relative units, RU) determined using Vero cell cytotoxicity assays. Biofilm formation was quantified in a 96-well microtitre plate assay with crystal violet staining.

Results: Geometric mean MICs (GM-MICs) differed between ribotype groups. Ribotype 027 isolates were significantly more resistant to ciprofloxacin (68.2 mg/L), moxifloxacin (32 mg/L), erythromycin (128 mg/L), and less susceptible to metronidazole (1.37 mg/L) than ribotypes 078 and 002 (P<0.001). Interestingly, GM-MIC of clindamycin was significantly higher for ribotype 002 (3.31 mg/L, 64% of isolates were resistant at 8 mg/L) than for ribotypes 027 (0.44 mg/L) and 078 (0.09 mg/L) (P<0.001). Ribotype 078 isolates were generally more susceptible as assessed by GM-MICs, although intermediate resistance to tetracycline (8 mg/L) was observed in 54% of isolates (4 locations). Maximum specific growth rates in BHIS were: 0.84 h^{-1} (002), 0.67 h^{-1} (027), and 0.36 h^{-1} (078), with median 72 h cytotoxin titres substantially greater in 078 isolates (3 RU) compared to 027 and 002 (1 RU). Ribotype 078 isolates produced substantially greater levels of biofilm than 027 and 002 groups.

Conclusion: There are marked differences between *C. difficile* ribotypes with regard to antimicrobial susceptibility, growth (biofilm and planktonic) and cytotoxin production. Ribotype 027 was generally more antibiotic-resistant; and ribotype 078 was less susceptible to tetracycline. Ribotype 078 produced substantially greater amounts of cytotoxin than the other ribotypes, despite little difference in culture biomass, and demonstrated increased biofilm formation, which could contribute to enhanced virulence if found *in vivo*. Ribotype 002 had the greatest μ_{max} and levels of resistance to clindamycin; this is now the most prevalent UK *C. difficile* clinical ribotype, and further studies are warranted to explain this emergence.

Keywords: *C. difficile,* antimicrobials, biofilm, ribotype, hypervirulence, cytotoxin.

Introduction

- Despite improved clinical management strategies for CDI, healthcare treatment costs remain high and have been estimated in the USA at $1.1-3.2 billion [1-2].
- *C. difficile* hypervirulence: attributed to both RT027 & RT078 due to increased CDI severity [3-4].
- Initially increased severity of RT027 infection was believed to be a consequence of quantitatively higher Toxin A and Toxin B production due to 18-bp deletion and a point mutation in *tcdC*
  - However, not all studies have reproduced elevated toxin production in RT027 when this strain has been compared to other prevalent RT [5]
- RT078 has a deletion distinct from that present in RT027 (39-bp) and a point mutation within *tcdC*
- Jhung et al observed lower toxin production by RT078 than RT027 [6], but higher toxin production that a comparator *C. difficile* group
  - However, experiments using the method described [3, 6] did not use routine culture media and used sub-optimal detection methods, which are lack sensitivity & specificity
- UK: national distribution of *C. difficile* RT is monitored by the *C. difficile* Ribotyping Network [7]

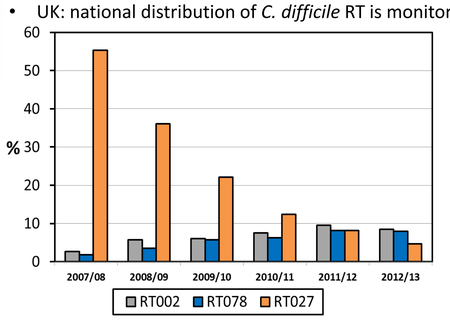

Figure 1. UK *C. difficile* RT prevalence 2007-2013 (data supplied courtesy of CDRN)

- RT027 has declined 50.6% in this period. RT078 and RT002 have gradually increased in prevalence by 6.1% and 5.8% respectively Little is known about the physiology of RT078 & RT002

Aim of this study: to compare RT027, RT078, and RT002 phenotypic characteristics: quantification of: cytotoxin production, biofilm formation, antimicrobial susceptibilities & maximum specific growth rates

Materials & Methods

***C. difficile* strains**

- UK strains: 11 each of RT002 (6 locations), RT027 (5 locations), and RT078 (5 locations)
- Controls: ATCC 700057 (ribotype 038) and E4 (ribotype 010, non-toxigenic)

Antimicrobial susceptibility testing

- Agar incorporation MICs on Wilkins-Chalgren agar, 10^4 cfu per spot [8]
- Antimicrobials: clindamycin (CLI), erythromycin (ERY), tetracycline (TET), metronidazole (MET), vancomycin (VAN), linezolid (LIN), ciprofloxacin (CIP), and moxifloxacin (MOX)

Biofilm quantification

- Culture medium: BHI broth + 0.1% (w/v) L-cysteine HCl + 0.5% (w/v) yeast extract (BHIS) [9]
- Quantification after 3 and 6 days with crystal violet staining (OD_{590}) after methanol extraction

Growth rate analysis & cytotoxin production

- BHIS overnight cultures (anaerobic, 37°C) were adjusted to OD_{600} = 0.10 in sterile medium
- Growth rate analysis: OD_{600} determined at time (h): 2, 4, 5, and 6 during exponential growth
- Cytotoxin production analysis:
  - Vero cell cytotoxicity assay of BHIS cultures (24 h, 48 h , and 72 h)
  - Cytotoxin titres expressed as log_{10} relative units (RU)

Statistical analysis

- Data were assessed for normality of distribution and homogeneity of variance using Minitab version 16 and SPSS version 20. For non-parametric data medians were compared using the Kruskal-Wallis test (significance set at P<0.05) with additional post-hoc testing.

Results

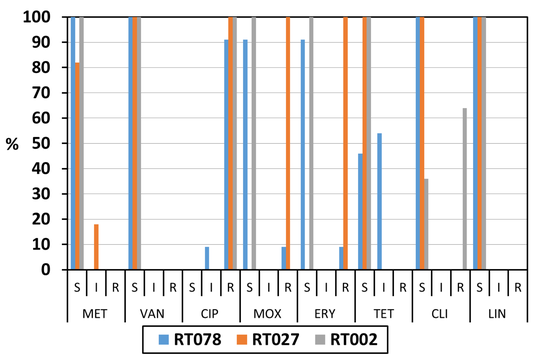

Figure 2. Antimicrobial susceptibility data for UK *C. difficile* RT002, RT078, and RT027 (N=11 each RT). S, susceptible; I, intermediate resistance; R, resistance using CLSI, EUCAST, or ECOFF breakpoints.

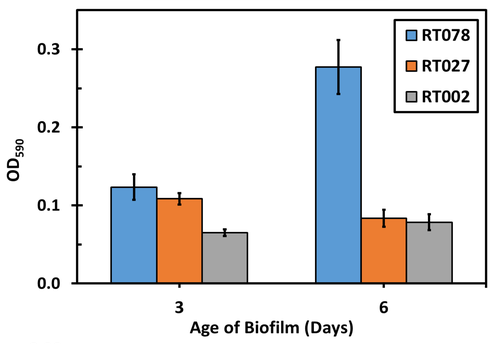

Figure 3. Mean (±SE) biofilm production (OD_{590}) in a microtitre plate assay with crystal violet staining after 3 and 6 days anaerobic incubation in BHIS of *C. difficile* RT078, RT027, and RT002 (11 strains each RT)

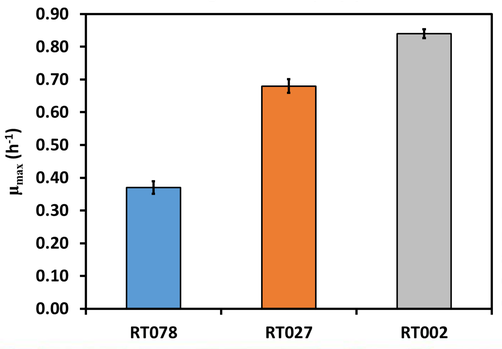

Figure 4. Maximum specific growth rates (μ_{max}) of *C. difficile* RT078, RT027, and RT002 (h^{-1}, mean ±SE) in BHIS during exponential growth (11 strains each RT)

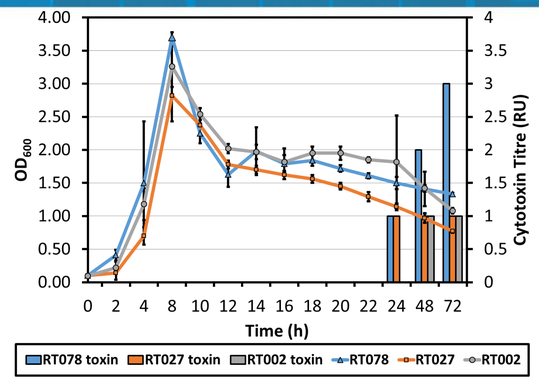

Figure 5. *C. difficile* RT078, RT027, and RT002 growth curves in BHIS (Mean ±SE) and cytotoxin titres (Median titre, RU). Eleven strains of each RT were assessed.

Results summary

Antimicrobial susceptibilities (Figure 2)

- RT027 strains were significantly more resistant to CIP (GM 68.2 mg/L), MOX (GM 32 mg/L), ERY (GM 128 mg/L), and less susceptible to MET (GM 1.37 mg/L) than ribotypes 078 and 002 (P<0.001).
- GM-MIC of CLIN was significantly higher for ribotype 002 (3.31 mg/L, 64% of isolates were resistant at 8 mg/L) vs. RT027 (0.44 mg/L) and RT078 (0.09 mg/L) (P<0.001).
- RT078 isolates were generally more susceptible but showed intermediate resistance to TET (8 mg/L, 54% of isolates).

Biofilm quantification(Figure 3)

- RT078 biofilm was 3-fold > RT027 & RT002 after 6 days (P<0.0001) of BHIS incubation
- RT027 and RT002 biofilm production was low and not significantly different (P=0.791)

Maximum specific growth rate (Figure 4)

- Median μ_{max} was statistically significantly higher for RT002>RT027>RT078 (P<0.0001) in BHIS; median values were 0.84>0.67>0.34 h^{-1} respectively

Cytotoxin production profiles (Figure 5)

- No substantial difference in peak OD_{600} was observed between RT groups
- Low-level cytotoxin activity was observed for RT027 and 078 earlier in growth curves but was below the lower limit of quantitation (data not shown)
- 1RU cytotoxin activity was present at 24h for RT078 and 027
- RT078 median cytotoxin titre at 72h was significantly greater than RT027 (P<0.001), but RT027 and RT002 titres did not differ significantly (P=0.936)

Discussion & conclusions

C. difficile RT078 and RT002 have increased in prevalence in UK CDI over the past 5 years, yet it is unclear whether there are phenotypic/genotypic traits of these RT that are facilitating these increases or whether this is a consequence of reducing RT027 CDI.

- RT027 was the most antimicrobial-resistant RT evaluated; indeed resistance in all isolates to ERY (but not CLIN) and CIP/MOX which reflects prior studies in RT078 [11]
  - Resistance mechanisms in RT027, RT078, and require further analysis
- RT078 produced significantly more cytotoxin than RT027 (and RT002) over 72 h incubation in BHIS, in contrast to previous reports [6]; however a more specific and sensitive detection method was used in addition to different media
- Additionally, biofilm formation in a 96-well microplate assay was significantly greater in the RT078 group, despite RT078 being non-flagellated [10] unlike RT027; suggesting that RT078 can form biofilms independently of flagella
- Interestingly , RT002 μ_{max} was significantly greater than RT027 and RT078; which may confer RT002 a competitive advantage against other *C. difficile* strains and the normal microflora. Further studies assessing the nutrient utilisation of RT002 may be warranted.
- Several phenotypic characteristics that could contribute to RT078 emergence have been described in this study and warrant assessment/validation using other model systems.

References

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