
https://doi.org/10.1016/j.anaerobe.2018.11.009
A series of three cases of severe *Clostridium difficile* infection in Australia associated with a binary toxin producing clade 2 ribotype 251 strain

Michael C. Wehrhahn, Caitlin Keighley, Jelica Kurtovic, Daniel R. Knight, Stacey Hong, Melanie L. Hutton, Dena Lyras, Qinning Wang, Rupert Leong, Tom Borody, Michael Edye, Thomas V. Riley

PII: S1075-9964(18)30201-4
DOI: https://doi.org/10.1016/j.anaerobe.2018.11.009
Reference: YANAE 1971

To appear in: *Anaerobe*

Received Date: 29 August 2018
Revised Date: 13 November 2018
Accepted Date: 26 November 2018


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
A series of three cases of severe *Clostridium difficile* infection in Australia associated with a binary toxin producing clade 2 ribotype 251 strain

Michael C. Wehrhahn\textsuperscript{a#}, Caitlin Keighley\textsuperscript{b}, Jelica Kurtovic\textsuperscript{c}, Daniel R Knight\textsuperscript{d}, Stacey Hong\textsuperscript{e}, Melanie L. Hutton\textsuperscript{f}, Dena Lyras\textsuperscript{f}, Qinning Wang\textsuperscript{b}, Rupert Leong\textsuperscript{g}, Tom Borody\textsuperscript{h}, Michael Edye\textsuperscript{i}, Thomas V Riley\textsuperscript{d, e, j, k}

\textsuperscript{a} Microbiology Department, Douglass Hanly Moir Pathology, Macquarie Park, NSW, Australia
\textsuperscript{b} Centre for Infectious Diseases and Microbiology Laboratory Services, Westmead, NSW
\textsuperscript{c} Gastrointestinal and Liver Unit, Prince of Wales Hospital, Randwick NSW
\textsuperscript{d} School of Veterinary & Life Sciences, Murdoch University, Murdoch WA 6150
\textsuperscript{e} School of Biomedical Sciences, The University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands, WA
\textsuperscript{f} Infection and Immunity Program, Monash Biomedicine Discovery Institute and Department of Microbiology, Monash University, Clayton, Victoria
\textsuperscript{g} Macquarie GI, Macquarie University Hospital, NSW
\textsuperscript{h} Centre for Digestive Diseases, Five Dock, NSW
\textsuperscript{i} Blacktown Mount Druitt Clinical School, Western Sydney University, NSW, 2148
\textsuperscript{j} Department of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands WA 6009
\textsuperscript{k} School of Medical & Health Sciences, Edith Cowan University, Joondalup WA 6027

\# Corresponding author: Michael C. Wehrhahn: email: mwehrhahn@dhm.com.au; address: 14 Giffnock Ave, Macquarie Park NSW 2113 Australia; phone: +612 98555287; fax: +612 98555650.
ABSTRACT (211 words)

Three patients with severe *Clostridium difficile* infection (CDI) caused by an unusual strain of *C. difficile*, PCR ribotype (RT) 251, were identified in New South Wales, Australia. All cases presented with severe diarrhoea, two had multiple recurrences and one died following a colectomy. *C. difficile* RT251 strains were isolated by toxigenic culture. Genetic characterisation was performed using techniques including toxin gene profiling, PCR ribotyping, whole genome sequencing (WGS), *in-silico* multi-locus-sequence-typing (MLST) and core-genome single nucleotide variant (SNV) analyses. Antimicrobial susceptibility was determined using an agar incorporation method. *In vitro* toxin production was confirmed by Vero cell cytotoxicity assay and pathogenicity was assessed in a murine model of CDI. All RT251 isolates contained toxin A (*tcdA*), toxin B (*tcdB*) and binary toxin (*cdtA* and *cdtB*) genes. Core-genome analyses revealed the RT251 strains were clonal, with 0-5 SNVs between isolates. WGS and MLST clustered RT251 in the same evolutionary clade (clade 2) as RT027. Despite comparatively lower levels of *in vitro* toxin production, in the murine model RT251 infection resembled RT027 infection. Mice showed marked weight loss, severe disease within 48 h post-infection and death. All isolates were susceptible to metronidazole and vancomycin. Our observations suggest *C. difficile* RT251 causes severe disease and emphasise the importance of ongoing surveillance for new and emerging strains of *C. difficile* with enhanced virulence.

Keywords

*Clostridium difficile*, ribotype 251, severe infection, fatal infection
**Introduction (Text: 2757 words)**

*Clostridium difficile* (also now named *Clostridioides difficile*) [1, 2] continues to be a significant cause of gastrointestinal disease worldwide. In the USA, the estimated numbers of *C. difficile* infections (CDIs) and deaths in 2011 were 453,000 and 29,300, respectively [3]. In a convenience sample of 1,364 *C. difficile* isolates from the same study, NAP1 (ribotype [RT] 027) strains accounted for half (49.5%) of those typed. In Australia, the molecular epidemiology of CDI differs from that seen in the Northern Hemisphere, and major outbreaks caused by RT027 have not occurred. In a survey of RTs of *C. difficile* circulating in Australia in 2010, the two most common RTs were RT14/020 (30.0%) and RT002 (11.8%), followed by RT054 (4.2%), RT056 (3.9%) and RT070 (3.6%). Several binary toxin (CDT)-positive isolates were detected including three RT027 (0.9%) isolates identified in NSW, one RT078 (0.3%) isolate in NSW, one RT127 (0.3%) in NSW, one RT251 (0.3%) in NSW and one RT244 (0.3%) isolate in Qld [4]. A later survey in 2012 showed continued predominance of RT014/020 group (25.5%) and RT002 (10.5%) with RT027 rarely seen. However, the proportion of RT244 had increased significantly to 2.4% and, while not a statistically significant increase, RT251 now comprised 1.1% of isolates [5]. *C. difficile* RT244 is a strain closely related to RT027 that caused severe mainly community-associated (CA) infections in both Australia and New Zealand [6-8]. In this report, we describe three cases of severe CDI caused by *C. difficile* RT251 that appeared in Australia at the same time as RT244 to raise awareness of the association of RT251 with severe CDI and to motivate ongoing studies about these strains.

**Case Reports**

**Case 1**

A 79-year-old female with scleroderma, multiple myeloma, diverticulosis, asthma and peripheral vascular disease, presented to hospital on 10/5/12 with increasing dyspnoea and
cough. She received 5 days of ceftriaxone and azithromycin for presumptive pneumonia. On 15/5/12, she developed diarrhoea which was thought to be due to the recent antimicrobials, and aperients given 3 days earlier. Her antimicrobials were promptly ceased. The diarrhoea initially improved but recurred on 20/5/12 in association with abdominal pain and an elevated white cell count (WCC) of 33×10⁹/L. On 21/5/12, intravenous meropenem and metronidazole were started to cover for the differential of diverticulitis, ischemic bowel (in the setting of new atrial fibrillation), pneumonia and CDI. On 22/5/12 she developed fever (37.9°C) and an abdominal computed tomography (CT) demonstrated colitis. The *C. difficile* tcdB (toxin B) gene was detected by PCR on stool from 21/5/12 and her C reactive protein (CRP) peaked at 182 mg/L on 23/5/12. Meropenem was changed to oral ciprofloxacin and clindamycin for a further 3 days for hospital acquired pneumonia, with metronidazole continuing for 1 week after the other antimicrobials were stopped (18 days treatment in total). Normal levels of WCC and CRP were demonstrated post-treatment, although her eosinophil count was elevated at 2.7×10⁹/L. The *C. difficile* strain was ribotyped and identified as RT251.

She subsequently suffered three further laboratory-confirmed episodes (*tcdB* gene PCR positive): the first on 11/6/12 (3 days after ceasing metronidazole) associated with fever to 38.0°C, peak WCC 44.5×10⁹/L and CRP 323 mg/L; the second on 11/8/12 (2 weeks after ceasing a 6-week tapering course of oral vancomycin) associated with peak WCC 23.7×10⁹/L and CRP 124 mg/L; and the third on 17/9/12 (12 days after ceasing a 4-week tapering course of oral vancomycin) associated with peak WCC 17.8×10⁹/L and CRP 90 mg/L. Ribotyping of the isolate from her second episode identified RT070, while the third episode was identified again as being caused by RT251. Because of presumed intolerance to prolonged vancomycin (abdominal pain associated with eosinophilia that resolved after cessation), and difficulties in obtaining rifaximin or fidaxomicin, faecal microbiota transplant (FMT) was arranged to treat the third episode. After 1 week of oral vancomycin, she received 250 mL of healthy donor
stool via colonoscopy and a second infusion via enema the following day. Normal bowel motions returned within 2 days and she had no further recurrences for 10 months until her death from an unrelated cause.

**Case 2**

A 22-year-old female university student presented with symptoms of a urinary tract infection to her general practitioner on 22/02/12. She was empirically commenced on norfloxacin but subsequently changed to amoxicillin on the 29/2/12 after culture identified *Escherichia coli* and an *Enterococcus* spp. Seven days into treatment, she developed ongoing diarrhoea and abdominal pain, and presented to an emergency department (ED) on 16/3/12. Despite a high WCC (15.6×10^9/L) she was discharged without specific therapy, however, she returned to the ED 5 days later with worsening diarrhoea. Toxigenic *C. difficile* was detected by cytotoxicity assay using Vero cells in addition to a positive *tcdB* PCR from her earlier presentation on 16/3/12 and she commenced a planned 2-week course of ciprofloxacin and metronidazole, showing recovery 4 days after treatment.

She subsequently suffered three laboratory-confirmed recurrences, the first two diagnosed by the same methods as the initial episode and the last by detection of *tcdB* by PCR: the first on 14/4/12 (6 days after ceasing metronidazole) that required a 3-day hospital admission; the second on 6/5/12 (12 days after ceasing metronidazole) and the third on 3/8/12 (5 days after ceasing a prolonged tapering course of oral vancomycin). Each episode was associated with diarrhoea, fever and elevated CRP. Ribotyping of her final isolate identified *C. difficile* RT251. Vancomycin was restarted but changed back to metronidazole for 1 month with no further presentations.

**Case 3**

A 32-year-old female pastry chef presented to hospital on 10/8/2015 with a 4-day history of fever (temperature 38°C), diarrhoea and abdominal pain. Her background included
laxatives for weight control, and attention deficit hyperactivity disorder (ADHD). She had received no antimicrobials in the preceding year. Her WCC and CRP were 17.5×10^9/L and 417 mg/L, respectively. She was commenced on oral ciprofloxacin followed by intravenous ceftriaxone. On 11/08/15, ongoing fever and the development of an acute abdomen led to the addition of intravenous metronidazole and gentamicin. She was transferred to a tertiary hospital where a CT of her abdomen demonstrated colitis without perforation. Her albumin had fallen to 11 g/L, and her WCC and CRP had risen to 37.4×10^9/L and 442 mg/L, respectively. Intraluminal vancomycin was administered and ceftriaxone was replaced by piperacillin-tazobactam. A laparotomy was considered and deferred because of apparent improvement.

On 12/08/15 she developed lactic acidosis with pH 7.1, lactate 7.3 mmol/L, acute renal failure and coagulopathy, and a positive C. difficile tcdB PCR result was received from 10-11/8/15. At laparotomy features of compartment syndrome with ischaemia were observed. Although very oedematous, the colon was intact, well perfused and appeared viable. A loop ileostomy and colonic lavage was performed. She returned from the operating theatre intubated and on inotropic support. Intravenous metronidazole and intraluminal vancomycin 2g/24 h infusion via the ileostomy was given. Whilst her inflammatory markers improved, her lactate increased and multi-organ failure ensued. On 13/08/15 she underwent a colectomy. A pan-enteric PCR panel confirmed C. difficile as the only pathogen. Histopathology confirmed pseudomembranous colitis and demonstrated no underlying inflammatory bowel disease or alternate pathology. Further resections of ischaemic bowel, multiple abdominal washouts and supportive care for massive bleeding and diffuse intravascular coagulation failed to improve her condition. After discussion with family, supportive care was withdrawn on 4/09/2015 and she expired. Ribotyping of her two isolates identified C. difficile RT251.
Material and Methods

Identification and characterisation of C. difficile

All three cases were associated with an elevated WCC >15×10⁹/L, colectomy, death or recurrence and were therefore defined as recurrent and/or severe disease [9]. Culture and identification of C. difficile, antimicrobial susceptibility testing (by the agar dilution methodology), PCR for tcdA, tcdB, cdtA and cdtB toxin genes and PCR ribotyping were performed as previously described [10]. Cytotoxin production from C. difficile was detected using a Vero cell cytotoxicity assay [11]. C. difficile control strains VIP 10463 (RT087), 630 (RT012) and R20291 (RT027) were included in these assays for comparative purposes.

Virulence in a mouse model

C. difficile spores from a RT027 strain (M7404) or a RT251 strain (ES1209) were prepared for mouse infection experiments as described by Carter et al. [12]. Animal handling and experimentation were performed in accordance with institutional guidelines (Monash University AEC no. MARP/2014/135). Male, C57BL/6J, 6-7 week old mice (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) were pre-treated with antibiotics in the drinking water for 7 days. On the day of infection, mice were switched to plain drinking water prior to infection or not with C. difficile M7404 or ES1209 spores (10⁵ spores/mouse) by oral gavage. Mice were monitored daily for signs of disease (weight loss, diarrhoea, behavioural and physical changes) and faeces were collected at 24 h post-infection to enumerate C. difficile spore load. The colonic and caecal tissues from each mouse were Swiss-rolled and sectioned transversely and stained with Periodic Acid Schiffs (PAS) and Alcian Blue [13]. Tissues were assessed for histopathological damage [12].

Whole genome sequencing and phylogenetic analysis

Following subculture of C. difficile isolates on blood agar for 24 h, genomic DNA was extracted using a Gentra Puregene Yeast/Bact. Kit [Qiagen, Hilden, Germany] and...
multiplexed paired-end (PE) libraries were generated using standard Nextera XT protocols [Illumina, CA, USA]. Strains were sequenced to a median depth of 68X on a HiSeq 2500 platform [Illumina] using 100 bp PE chemistry. Raw reads were deposited in the European Nucleotide Archive (study PRJEB13264, accessions ERS1102068-71). After trimming for quality (minimum Phred score of Q30) and Nextera adapter content, multi-locus sequence type (MLST,ST) was inferred in silico using the pubMLST database [http://pubmlst.org/cdifficile] compiled within the short read sequence typing program SRST2 [14]. Core-genome single nucleotide variant (SNV) analysis was performed as previously described [15] with C. difficile R20291 (MLST clade 2, ST1, accession FN545816) used as a reference chromosome for read mapping. A final set of 8217 core-genome SNVs in ‘clonal frame’ was used for maximum-likelihood phylogenetic analysis [15].

Results

In vitro antimicrobial susceptibility

All RT251 isolates were susceptible to metronidazole (MIC range 0.25-0.5 mg/L), vancomycin (1-2 mg/L), rifaximin (0.015-0.03 mg/L), fidaxomicin (0.06-0.5 mg/L), amoxicillin/clavulanate (0.5mg/L), meropenem (2mg/L) and moxifloxacin (2mg/L). Low level clindamycin (8 mg/L) and erythromycin (≥256 mg/L) resistance was observed only for isolate ES 1207 (Case 1) mediated by a methylase encoding \textit{ermB} gene.

Cytotoxicity assays

Toxin gene PCR confirmed the presence of toxin A (\textit{tcdA}), toxin B (\textit{tcdB}) and binary toxin (\textit{cdtA} and \textit{cdtB}) genes. All C. difficile RT251 isolates produced relatively lower amounts of cytotoxin compared to strains VPI 10463, 630 and R20291 with an average 90% cytopathic effect (CPE) toxin titre of 10^-5 dilution (p ≤ 0.05). VPI 10463 and R20291 produced the highest level of cytotoxins with titres of 10^-9 and 10^-8 dilutions, respectively.
Interestingly, 630 produced an average 90% CPE toxin titre of $10^{-7}$ dilution, which was 100-fold higher than the RT251 isolates despite being recognised as a low toxin producer [16]. All C. difficile RT251 isolates contained a wild-type tcdC gene and no deletions.

**In vivo virulence**

As previously described [12], infection of mice with the C. difficile M7404 (RT027) strain resulted in consistent colonisation and rapid disease onset, with signs of diarrhoea by 24 h, marked weight loss, and decreased activity observed in 80% of mice by 36 h post-infection (Figure 1). Similarly, infection with a C. difficile RT251 isolate (ES1209) resulted in severe, fulminant disease albeit with slightly delayed presentation (36 h vs 24 h).

Typical features of disease were observed in colonic and caecal tissue from all RT251-infected animals (Figure 2). This included extensive damage to the epithelial surface, crypt hyperplasia, goblet and epithelial cell loss and severe inflammation, as seen by significant neutrophil influx and oedema. These features have been described before for infections resulting from toxigenic and virulent strains of C. difficile and were not present in uninfected tissue [12].

**RT251 strain relatedness based on MLST and evolution in the core-genome**

*In silico* MLST showed that all RT251 isolates belonged to clade 2 sub-lineage ST231 (Figure 3) and share a close evolutionary relationship with other clade 2 virulent sub-lineages ST41 (RT244) and ST1 (RT027) showing allelic conservation in 5/7 and 3/7 housekeeping genes, respectively (data not shown). SNV analysis on the non-repetitive, non-recombinant core-genome provided ultra-fine scale discrimination of the 4 RT251 isolates (Figure 3). Isolates ES1209 and ES1205 (case 3, August 2015) were indistinguishable in their core-genome (0 SNVs difference) and differed by a single variant from ES1213 isolated three years earlier (case 2, August 2012). ES1207 (case 1, June 2012) differed by 4-5 SNVs in its core-genome from isolates from cases 2 and 3. Based on previous estimations of the C.
difficile molecular clock (1.47×10⁻⁷ to 5.33×10⁻⁷ mutations per site per year, equating to 1-2 SNVs per genome per year) a cut-off of 0-2 SNVs has been proposed as a signature of a recent clonal transmission event [15, 17]. In this context, the three isolates comprising cases 2 and 3 would appear to be RT251 clones originating from a single common source. Conversely, the 4-5 SNVs between case 1 and cases 2 and 3 rules out direct transmission or a single common source, but does still show a very recent evolutionary history.

Discussion

For many years, virulence factors for C. difficile were regarded as having a negligible role in disease, with disease severity dictated largely by host factors. C. difficile RT027 was the first strain of C. difficile to be associated with increased morbidity and mortality [18], although this finding continues to be debated [19]. Subsequently, several other strains have also caused CDI with apparent increased morbidity and mortality, such as RT078 [20], RT176 [21] and, more recently in Australia and New Zealand, RT244 [6-8]. The C. difficile RT251 strains isolated in the three cases described here were responsible for complicated recurrent disease, and death in one case. Using a mouse model of CDI, we showed that a RT251 strain can cause severe disease, comparable to that caused by a RT027 strain. The in vivo pathogenicity observed in the murine model corroborated the disease presentation observed in the clinical cases described.

Interestingly, toxin titres for this RT were unexpectedly lower than those observed for the control strains 630 and R20291, suggesting RT251 may possess other virulence factors. These factors in combination with favourable host conditions may lead to the potential for increased disease severity. In addition, earlier studies have shown that low toxin production in vitro does not necessarily correlate with in vivo virulence and this appears to be a poor measure for determining the potential of a given isolate to cause severe disease [22-24]. Some studies have shown that binary toxin-producing strains of C. difficile have the ability to
cause severe disease on their own and subvert the host immune response in conjunction with toxins A and B [25, 26]. The synergistic interactions between toxins and the presence of other virulent factors of RT251 require further research.

For *C. difficile*, at least 5 evolutionary clades are currently recognised and some show a high degree of geographical tropism; clade 1 (commonly found in Europe), clade 2 (North America), clade 4 (Asia) and clade 5 (Australia) [17, 27]. *In silico* MLST identified RT251 as ST231 and belonging to clade 2 along with other “hyper-virulent” strain lineages RT027, RT244 and RT176. With its likely origin in North America this begs the question, “How did it get to Australia?” *C. difficile* RT251 (previously identified as type C) has been isolated from both production animals and meat destined for human consumption in North America [28, 29], and fresh pork is imported from North America before processing in Australia for domestic consumption [30]. Should there be any contamination of this pork with *C. difficile* spores, then neither routine meat processing, preservative treatment nor recommended cooking temperatures (160°F/71°C) would destroy them [31-33]. Other possible sources of these strains of *C. difficile* in Australia are onions which were imported into Australia from California in 2011-12 following a drought on the east coast of Australia [34]. A recent Australian report showed that toxigenic *C. difficile* could be commonly isolated from Australian root vegetables such as onions and potatoes [35] and, in a very recent publication from Slovenia, *C. difficile* RT244 was obtained from one potato sample of USA origin, confirming that root vegetables contaminated with soil are a potentially important source of *C. difficile* [36]. In addition, the contamination of gardens in general with compost containing *C. difficile*, and lawns [37] may be an emerging issue. These findings further emphasise the ubiquitous nature of *C. difficile* spores and the likelihood of CDI resulting from the consumption of contaminated food or from contact with *C. difficile* in the environment.
SNV analysis is a powerful tool for investigating fine-scale genetic variability in bacterial genomes [38]. Here, it was able to substantiate transmission events for two of the three CDI cases and showed a very close evolutionary relationship for both cases to the third. As anticipated, isolates ES1209 and ES1205 (both case 3 and isolated 1 day apart) were indistinguishable in their core-genome. Notably, however, these clones differed by only 1 SNV from ES1213 (case 2), a CDI episode occurring 3 years earlier. The finding that possible clonal acquisition occurred 3 years apart is likely due to *C. difficile* spore dormancy, where cells remain genetically quiescent and evolution is slowed significantly or even completely arrested [39]. Case 1 may have originated from an earlier introduction into Australia and studies are underway to identify its relatedness to other RT251 strains detected in our region as far back as 2010 when a single isolate of RT251 was recovered in New South Wales [4]. It is possible that there have been multiple importations into Australia of RT251 clones from North America and RT251 may represent a persistent source of *C. difficile* in the community.

These three cases are notable for several reasons other than the strain of *C. difficile* responsible. The first two cases had multiple recurrences occurring within 14 days of cessation of antibiotic therapy. The first case was in an elderly lady with multiple comorbidities and therefore it is not surprising that her course was severe especially given that she was treated with several antimicrobials to cover a range of diagnostic possibilities. In cases 1 and 2, the delayed use of vancomycin may also have led to increased severity, however, vancomycin was avoided in the initial episode due to good early response to metronidazole, limited availability of oral vancomycin (which is also unsubsidised for initial episodes of CDI in Australia) and recommendations from local guidelines to limit the use of vancomycin to prevent rising infection rates of vancomycin-resistant enterococci. FMT was well-tolerated in case 1 and effective in terminating her recurrent disease and earlier use
deserves consideration [40]. PCR ribotyping played an important role as a rapid typing method to differentiate if the infection was due to relapse or re-infection with a different strain as was seen in the second recurrence in case 1. These three cases also contrast the “traditional” CDI, occurring in elderly, multiply co-morbid patients who develop infection in hospital, with the “new” CDI, occurring in young previously healthy individuals who acquire infection in the community.

Notwithstanding these findings, there remain limitations which prevent us from concluding with certainty that RT251 strains cause more severe disease than other ribotypes of *C. difficile*. In all three cases, there was some delay in the recognition that CDI was responsible for the clinical presentation of each patient. In case 2, it is possible that the early episodes were due to other ribotypes, as only the final isolate was ribotyped. It was initially thought that there were relatively small numbers of infections caused by *C. difficile* RT251 in Australia as compared to RT244 which had increased to become the 3rd most prevalent ribotype in Australia [41]. This was because *C. difficile* RT244 was much easier to detect in the laboratory than RT251 due to its PaLoc mutations similar to RT027 and therefore signalling on the Xpert PCR platform [7, 8]. Consequently, clinical and other data pertaining to RT251 was lacking and the proportion of cases causing severe disease was not known. We have subsequently amassed over 100 cases of CDI in Australia caused by RT251 and are currently conducting a case-control study to look at severity of disease. Demonstration of severe disease in a mouse model does not necessarily mean severe disease will occur in humans, although the mouse model used does correlate well with human disease [12]. In addition to further work examining the clinical characteristics of a larger group of RT251 infections, investigations have been undertaken to genotypically and phenotypically characterise RT251 strains isolated in Australia and North America to ascertain virulence factors and the evolutionary history of this lineage. These indicated Australian *C. difficile*
RT251 strains share a common ancestry with RT027 and are likely of North American origin [42].

Acknowledgements: We are grateful for the facilities, and scientific and technical assistance of the Monash Histology Platform, Department of Anatomy and Developmental Biology, Monash University.

Declarations of Interest: None.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.
References:


11. Bowman RA, Riley TV, Isolation of Clostridium difficile from stored specimens and comparative susceptibility of various tissue culture cell lines to cytotoxin. FEMS Microbiol. Letts. 30 (1986) 31-5.


33. Lim SC, Foster NF, Riley TV, Susceptibility of *Clostridium difficile* to the food preservatives sodium nitrite, sodium nitrate and sodium metabisulphite, Anaerobe 37 (2016) 67-71.


Figure 1. The RT251 strain ES1209 is virulent in mice. C57BL/6J mice (five per group) were either uninfected (black circles) or infected with $10^5$ spores of *C. difficile* strains ES1209 (RT251; black triangles) or M7404 (RT027; grey squares). Faecal spore load at 24 h post-infection was determined by plating (a). Data are presented as CFU/gram faeces ($\log_{10}$), with each point representing a single mouse. Horizontal bars represent the mean ± S.E.M. of
spore count determinations. The dotted line represents the limit of detection of the assay. Mice were monitored daily for weight loss (b) and survival is presented as a Kaplan-Meier survival curve (c). Data represent the mean results ± S.E.M.

**Figure 2. Infection with the RT251 strain ES1209 causes extensive damage to the cecum and colon of infected mice.** Representative images of PAS-Alcian blue stained colonic and caecal tissue from uninfected mice or mice infected with either a RT251 (ES1209) or RT027 (M7404) strain of *C. difficile*. Red brackets (l) indicate crypt hyperplasia, arrow heads (^) represent surface epithelial damage and asterisks (*) represent oedema and inflammation. Scale bars (200 µm) are shown in yellow.
Figure 3. RT251 strain relatedness. (A) Pairwise core-genome SNV analysis for 4 C. difficile RT251 strains isolated from 3 independent cases in Australia. Relative SNV distance of case strains to C. difficile reference genome R20291 also shown (RT027, ST1, GenBank accession FN545816). Red numbers indicate signatures of clonal transmission/point source origin (0-2 SNVs). (B) Graphical representation of 16S–23S rRNA intergenic spacer region (ISR) banding patterns for clade 2 binary toxin positive RTs 251, 244 and 027.
Highlights

- Three cases of severe Clostridium difficile infection caused by Ribotype 251
- Mouse model confirms RT251 causes a rapidly fatal disease comparable to RT027
- RT251 belongs to the same evolutionary clade as RT027
- Arrival of RT251 in Australia possibly through imported food products