Clostridium difficile: from obscurity to superbug

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ABSTRACT

According to the UK media and popular press, *Clostridium difficile* is now a fully fledged member of that notorious but ill-defined group of microorganisms portrayed to the general public as superbugs. Following the trail blazed by methicillin-resistant *Staphylococcus aureus* (MRSA), *C. difficile* has made the transition from being an obscure anaerobic bacterium, mainly of interest to specialist anaerobic microbiologists, to that of an infamous superbug responsible for outbreaks of hospital-acquired infection that commonly result in serious disease and death. This review tracks the rise in scientific knowledge and public awareness of this organism.

KEY WORDS: Clostridium difficile.
Hospital-acquired infection.
Nosocomial infection.

Introduction

Clostridium difficile is an anaerobic Gram-positive, spore-bearing rod (Fig. 1) that was first described by the American workers Hall and O'Toole[1] who were studying the microbial flora of the meconium and faeces of newborns. Originally, it was named *Bacillus difficilis* owing to the difficulties experienced in culturing it using the technologies available in 1935. Furthermore, because it is an anaerobe, the sentiment that it is difficult to isolate and identify persists in many currently practising biomedical scientists who perceive that culturing *C. difficile* from faeces is complicated and can only be done in a reference laboratory. This belief has led to a widespread inability (particularly in UK diagnostic bacteriology laboratories) to culture faeces to obtain the isolates of *C. difficile* that are necessary to investigate their local epidemiology of *C. difficile* infections and also to contribute to national surveillance.

As *B. difficilis* was an obligately anaerobic Gram-positive, spore-bearing bacillus, it was classified subsequently as belonging to the genus *Clostridium*, and the species name was changed to *difficile*. Much later, debate ensued among microbiologists as to how to pronounce the species name. Hardened Latin scholars poured scorn on the French-sounding phonetic 'diffy-seel', but even they disagreed over the pronunciation of the last four letters, which can be pronounced phonetically as 'killy' or 'chilly'.

Hence, about 15 years ago, the author suggested at an international meeting in the USA that the various camps agree to disagree, and to abbreviate the species name (when using familiar terms) to 'diff'. Thus, the term C. diff was coined and this is the commonly used familiar name used by those who work with the organism, as well as those in the media who find long, complicated Latin names rather indigestible.

For several decades after its discovery, *C. difficile* made only fleeting appearances in the scientific literature. For example, McBee[2] isolated *C. difficile* from the intestine of a Weddel seal, and Smith and King,[3] who looked specifically for reports of *C. difficile* in human infections, noted eight incidents of extra-intestinal infection in which they concluded it was not playing a pathogenic role.

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Recognition of *C. difficile* as a pathogen

Although the 1960s and early to mid-1970s were within the antibiotic era, the phenomenon of antibiotic-associated diarrhoea, although recognised, was not perceived as sufficient of a problem to warrant much research into its causation. It was not until 1974 that three coincidental studies provided the evidence from which *C. difficile* was shown to be an important cause of antibiotic-associated disease in man.

In the USA, Green[4] described a cytotoxin that was present in the stools of guinea pigs that had developed diarrhoeal disease after receiving penicillin. Meanwhile, Tedesco *et al*.[5] found a significant association between patients who had

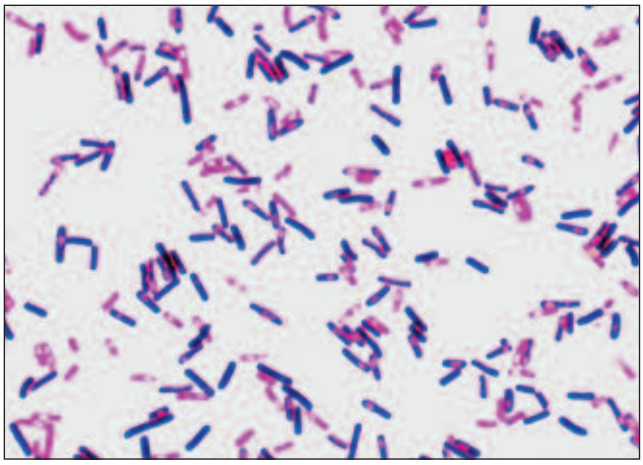

Fig. 1. Gram stain of *C. difficile* showing spores.
See this image in colour at www.bjbs-online.org

been treated with the antibiotic clindamycin and the development of pseudomembranous colitis (PMC), which resulted in the term clindamycin-associated colitis being coined in the USA. At this stage, neither worker knew the aetiology of their observations.

Meanwhile, in England, Hafiz, a PhD student studying under Professor C. L. Oakley in Leeds, was completing his thesis on *C. difficile*.[6] However, he was totally unaware that the organism he was studying was responsible for the symptoms described in humans and guinea pigs by Green and Tedesco. A few years later, Bartlett *et al*.[7] described a clindamycin-induced colitis in hamsters, and isolated an unidentified *Clostridium* species from the faeces of symptomatic animals that was believed to be the cause of their symptoms. This was identified subsequently as *C. difficile*.

Larson *et al*.[8] then demonstrated that a cytotoxin could also be detected in the faeces of five out of six patients with histologically proven PMC. Other studies soon followed and these provided confirmation that *C. difficile* was a cause of antibiotic-associated diarrhoea and colitis in man.[9,10] Thus, in the late 1970s, a new bacterial human intestinal pathogen was recognised. Pathogenesis was due to the production of two large toxins (A and B). Toxin A was shown to be an enterotoxin, with only minor cytotoxic properties, while toxin B proved to be powerful cytotoxin. Toxin-negative strains were also recognised and were considered to be non-pathogenic.

Here, it is apposite to consider the role of *C. difficile* toxins from a different perspective. The organism and the exotoxins it produces evolved long before man invented antibiotics. The toxins were metabolites that probably evolved to give the organism a selective advantage to gain nutrition from a particular substrate or to compete with other organisms for a particular anaerobic niche (e.g., the gastrointestinal tracts of various animal species). Thus, it is an unfortunate coincidence that by developing antibiotics that disturb the normal mammalian intestinal flora, man accidentally created a niche environment in which this normally harmless environmental organism could become pathogenic.

In summary, and not for the first time, it is man's intervention that has created the problem, and therefore *C. difficile* infection can be regarded as a truly iatrogenic disease.

Diagnostic challenges

Once *C. difficile* had become a recognised pathogen responsible for a range of enteric symptoms, from mild diarrhoea to life-threatening conditions such PMC, toxic megacolon and bowel perforation, it presented a diagnostic challenge to hospital microbiology laboratories. As the presence of either of the toxins – A (the enterotoxin) or B (the cytotoxin) – in the stool was considered as proof of infection, many diagnostic laboratories with virology departments chose to use tissue culture bio-assay techniques that were uniquely sensitive to the cytotoxin B. This involved making a filtered extract of the faeces under test and adding it to a monolayer of mammalian cells (e.g., Vero, MRC5 or HeLa) in tissue culture. Any cytopathic effect then had to be neutralised with either *C. difficile* or *C. sordellii* antitoxin to prove it was due to *C. difficile* toxins in the stool.

Commercial diagnostic companies recognised a niche market and soon developed kits for *C. difficile* toxin detection in stools. Initially, these were designed to detect only toxin A, and were based either on an immunochromatography principle, in which a coloured band indicates a positive result, or on an enzyme-linked immunosorbent assay (ELISA) format. Eventually, the ELISA kits proved popular with larger laboratories, as they were based on a microtitre well format that could be used in batches of any size, depending on workload.

Studies demonstrated acceptable levels of sensitivity and specificity,[11–13] and thus these methods largely have replaced the use of tissue culture in most laboratories in the UK. Later, research into the strains causing disease in the UK and abroad identified strains causing outbreaks that only produced toxin B,[14] and therefore kits have been adapted to include assays for toxin B to detect infections with A-negative/B-positive strains that would go undiagnosed using a kit designed to detect only toxin A. Other diagnostic targets for commercial kits included the semi-specific *C. difficile* enzyme glutamate dehydrogenase, and some more recent commercial kits contain both.

Recently, rapid molecular techniques such as real-time polymerase chain reaction (PCR) methods have been described; however, their widespread use in diagnostic laboratories is some way off at present.[15]

Once the optimal testing method has been chosen, one must then decide when to test. Factors include stool consistency, age of the patient, history of antibiotic treatment, in-patient or out-patient status and clinician request. A fairly recent initiative on this diagnostic dilemma saw proposal of the three-day rule. This suggests that if a patient has been in hospital longer than three days prior to developing diarrhoea or colitis, infection is very unlikely to be due to the common gastrointestinal species of *Salmonella*, *Shigella* or *Campylobacter*, and much more likely to be due to one of the agents of antibiotic-associated diarrhoea, of which *C. difficile* is the most common cause.[16]

If one looks at the figures for *C. difficile* toxin-positive stools in England and Wales (Fig. 2) per annum, from the 1980s to the present day, one is struck by the inexorable rise in cases, year on year. The increases seen in the 1980s could have been due in part to increased detection, as diagnostic laboratories gradually increased their repertoire of tests to

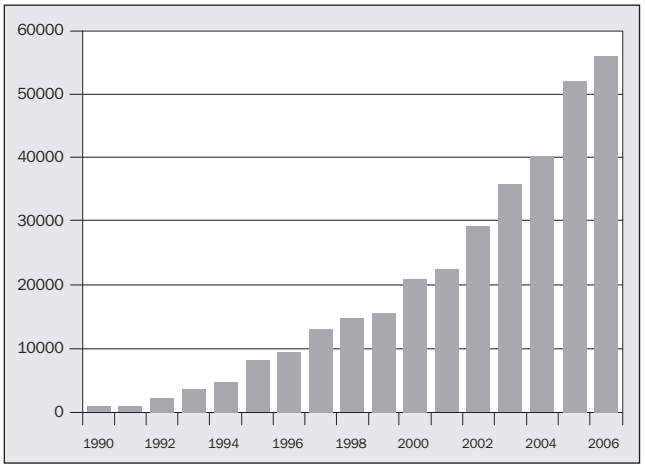

Fig. 2. Faecal toxin-positive reports 1990–2006 for England and Wales.

include *C. difficile* toxin detection and sometimes culture of the organism. However, the 50-fold increase since the early 1990s cannot be explained by this alone, and must represent a true rise in disease incidence.

Culture of *C. difficile* for diagnostic purposes decreased in importance as it became obvious that hospital patients could become colonised with the organism without showing signs of disease. Therefore, most busy laboratories chose to abandon the cultural methodology, not realising that by doing so they jeopardised their future ability to obtain isolates for outbreak investigation and susceptibility testing.

Typing

Once it became apparent that *C. difficile* infections were transmissible from patient to patient within a ward or hospital, it became of interest to try to type isolates to determine if cross-infection had taken place. Early typing methods were developed ostensibly to understand the epidemiology of *C. difficile* infection at a local level. Many of these investigations found evidence that a single strain was responsible for a number of cases in their hospital, thus confirming that *C. difficile* disease could be a cross-infection problem.

It soon became apparent, however, that while these methods were fine for local use, there was a need for typing schemes that could be applied to further our understanding of the epidemiology of *C. difficile* disease on a wider scale. To facilitate this, comparisons between typing schemes were performed, and Mulligan *et al*.[17] found good correlation between the types recognised by plasmid profiling, serotyping and polyacrylamide gel electrophoresis (PAGE) of cell-surface antigens and immunoblotting.

Molecular typing methods now have largely replaced phenotypic methods and are generally regarded as superior in terms of the stability of marker expression and provide greater levels of typeability. Several such molecular methods have been applied to *C. difficile*.

Plasmid profiling proved largely unsuccessful due to the sparse distribution of these extra-chromosomal genetic elements within the species, but analysis of chromosomal DNA of *C. difficile* was attempted by Kuijper *et al*.,[18] who used whole-cell DNA restriction endonuclease analysis (REA) using *Hind*III in an investigation that demonstrated cross-infection between two patients in the same room. Restriction endonuclease analysis is a highly discriminatory and reproducible method; however, it is a technically demanding procedure and very laborious, especially for large numbers of isolates.

Restriction fragment length polymorphism (RFLP) is an alternative genotypic method that involves initial REA digestion with infrequent cutting enzymes, followed by gel electrophoresis and Southern blotting with selected labelled nucleic acid probes to highlight specific restriction site heterogeneity. However, RFLP is also a labour-intensive method and thus REA/RFLP methods generally have been superseded by methods based on PCR techniques to amplify certain parts of the genome that may be discriminatory.

Arbitrarily primed PCR (AP-PCR) is a genotypic method that permits the detection of polymorphisms within the target genome without prior knowledge of the target nucleotide sequence. A closely related method called random amplified polymorphic DNA (RAPD) commonly uses two oligonucleotide primers that are short in length (about 10 bp) and also of arbitrary sequence. Barbut *et al*.[19] evaluated an RAPD method using two 10-bp primers in an investigation of AIDS patients with antibiotic-associated diarrhoea. The same PCR profiles were found in 25 isolates from 15 patients, suggesting infection with the same strain.

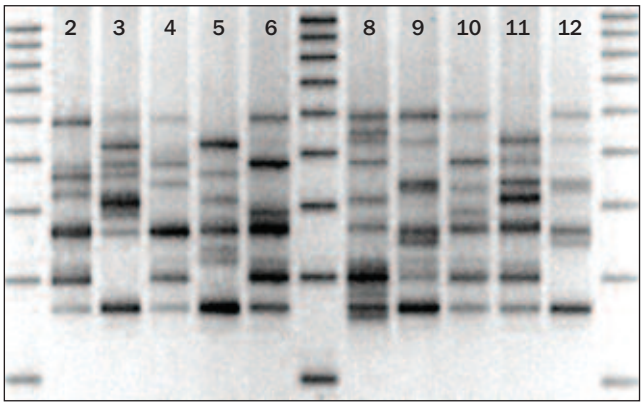

Fig. 3. 16S-23S rRNA spacer region PCR gel showing 10 distinct PCR ribotype profiles (lanes 2–6 and 8–12).

Polymerase chain reaction ribotyping uses specific primers complementary to sites within the RNA operon, and was first applied to *C. difficile* by Gurtler,[20] who targeted the amplification process at the spacer region between the 16S and 23S ribosomal RNA (rRNA) regions. *C. difficile* was shown to possess multiple copies of the rRNA genes, which not only varied in number between strains but also varied in size between different copies on the same genome. This approach was simplified by Cartwright *et al*.,[21] who applied it to 102 isolates obtained from 73 symptomatic patients. Using the same primers as Gurtler, they were able to separate the PCR fragments of a similar size range using straightforward agarose gel electrophoresis instead of denaturing PAGE. This approach was adapted further for routine use by O'Neill *et al*.,[22] who improved the methodology by greatly simplifying the DNA extraction method. Also, using modified primers, this method produced amplicons of 250–600 bp in length that could be separated by straightforward agarose gel electrophoresis (Fig. 3).

Discriminatory power was compared to Delmee's serogroups and gave different banding patterns for each of the 19 serogroups. This method was chosen by the UK Anaerobe Reference Laboratory (ARL) in Cardiff, which has provided a *C. difficile* typing service for the UK since 1995. From over 10,000 isolates from all sources examined, a library has been constructed from over 180 distinct PCR ribotypes.

This Cardiff ARL PCR ribotyping method uses a three-figure nomenclature ascribed to each distinct pattern of amplicons (e.g., type 001, etc). Investigations performed in Cardiff in the late 1990s discovered type 001 as the cause of many hospital outbreaks, although a true nationwide distribution was not determined because sampling was not uniform across the country.

This method is the current gold standard to which newer methods are compared.[23] Recently, newer molecular methods have been applied that are able to differentiate subtypes within a given ribotype (e.g., type 027),[24] and this should advance our epidemiological knowledge of this important strain.

National surveillance of *C. difficile* strains causing disease

Until quite recently, relatively little was known about the national distribution of strains of *C. difficile* circulating in hospitals in individual countries. Probably the most comprehensive national surveillance data have come from the surveillance study set up by the Department of Health and the Health Protection Agency in England, which was performed by the UK Anaerobe Reference Laboratory in Cardiff. This study of the strains causing infections in England began in 2005 and yielded 881 isolates on a random sampling basis from symptomatic patients in hospitals throughout England. Typing studies revealed that 75% of infections were caused by just three strains, identified as PCR ribotypes 001, 027 and 106, in roughly equal proportions.

Strains originating from GP patients and controls in England show a different distribution of PCR ribotypes compared to those found in English hospital patients. The most predominant strain in a community-based study that yielded 390 isolates was PCR type 010 (non-toxigenic), which accounted for 15.9% of isolates.[25] This indicates that certain strains seem to be adapted to the hospital environment and may even be selected for by local environmental pressures in the hospital.

In Europe, the ESCMID Study Group on *C. difficile* (ESGCD), which was founded by the author, has been established to focus on the problem of *C. difficile* infection from a European perspective. No doubt, this and other typing studies of *C. difficile* will play a key role in ongoing attempts to understand the global epidemiology of this nosocomial pathogen and its associated disease.

Stoke Mandeville outbreak and PCR ribotype 027

The PCR ribotype 027 was assigned as the 27th banding pattern recognised using the PCR ribotyping primers of O'Neill *et al*.[22] in the Anaerobe Reference Laboratory in Cardiff. It was assigned to an isolate called CD196, which originated from Professor Popoff's collection of *C. difficile* isolates sent to Cardiff from Paris in 1998. Only two other sporadic examples of this strain were seen in the UK in the next four years, and it was considered to be a rare and unimportant strain.

In March 2004 an outbreak investigation requested by Stoke Mandeville Hospital revealed that type 027 was the predominant strain in an outbreak that spanned nearly two years. This came to the attention of the media and sparked a government enquiry. Subsequent collaborative investigations[26] revealed that type 027 was indistinguishable from the strain that had caused outbreaks in America and eastern Canada, where it was known as NAP1 or BI using the different typing methods of pulsed-field gel electrophoresis (PFGE) and REA, respectively. These outbreaks predated the Stoke Mandeville episode and thus speculation suggested that type 027 may have been imported from North America, although this cannot be proved.

What is now known is that within weeks of the Stoke Mandeville outbreak, type 027 cropped up many other hospitals and has spread rapidly around England, as revealed by subsequent outbreak investigations and surveillance studies. Thus far, the ARL in Cardiff has tracked this strain to over 100 hospitals in England, eight in Wales and two in Scotland. Recently, studies in Europe have tracked its progress to The Netherlands, Belgium, France, Germany, Finland, Norway and Sweden. There has also been a single case in Vienna, Austria, in a British tourist who became ill while taking antibiotics for bronchitis after visiting her sick father in an English hospital known to have cases of infection due to type 027.

Although much of the evidence is anecdotal, type 027 is generally associated with increased severity of disease, which is believed to be due to increased levels of toxin production. Excess toxin production was believed to be due to a defective toxin-regulating gene called *TcdC*, as an 18 bp deletion was found in the sequence.[26] However, several other ribotypes possess similar and even larger deletions in this region and these are not associated with outbreaks or severe disease (personal observation). The over-production of toxins by type 027 is now thought to be due to a single point mutation, resulting in a premature stop codon and therefore a truncated and ineffective regulatory TcdC protein.

C. difficile in the media

The first mention of a *C. difficile* outbreak to make UK headlines was in the winter of 1991–92, when a least 17 patients died in an outbreak in a hospital in north Manchester. After it had made the local headlines, the local member of parliament raised the matter in the House of Commons, and an enquiry ensued under the auspices of the Public Health Laboratory Service and the Department of Health, which resulted in a report published in 1994.[27] Thereafter, although records show ever-increasing levels of disease in our hospitals (Fig. 2), the lack of a major incident prevented further press coverage, and *C. difficile* largely settled into obscurity.

Twelve years passed, during which *C. difficile* was confined to areas of specialist scientific interest only, although outbreaks were becoming increasingly common to those healthcare professionals concerned with hospital-acquired infections. In the meantime, the press had discovered MRSA

Fig. 4. Press headlines surrounding the Maidstone HCC Report in October 2007.

and the term 'superbug' was added to the English lexicon.

The summer of 2005 saw huge press coverage of the *C. difficile* outbreak at Stoke Mandeville hospital, with echoes of the long-forgotten Manchester outbreak. Again, the local MP tabled questions for the Prime Minister, asking why patients were dying of this disease in a hospital in his constituency. Obviously, politicians had forgotten the recommendations given in the 1994 report, as once again the government launched an enquiry, this time performed by The Healthcare Commission (HCC). Unlike the 1991–92 outbreak, advances in molecular typing of *C. difficile* revealed that a new strain called type 027 was responsible for the outbreak, and, following publication of a paper in *The Lancet* that showed it produced more toxins than other strains *in vitro*,[26] the press quickly dubbed type 027 the "virulent new superbug".

About a year later, in July 2006, the HCC report on the Stoke Mandeville outbreak was published, accompanied by renewed press interest. The report was critical of hospital management and several members of the trust management team resigned. This highlighted the importance of *C. difficile* infections to hospital trust managers hitherto preoccupied with meeting government targets that sometimes compromised recommended infection control measures.

Certain broadsheet investigative journalists followed up the initial story and published figures of cost estimates for *C. difficile* disease to the NHS, noting also the rise in the incidence of *C. difficile* infections in UK hospitals. More recently, in September 2006, *The Times* carried an article about *C. difficile* infections at a hospital in Maidstone, Kent, and just over a year later another HCC enquiry was published and received massive press coverage. The report was equally damning of trust managers (Fig. 4).

This outbreak was even larger than the one at Stoke Mandeville, with at least 90 deaths among a total of over 1100 cases over two and a half years directly attributable to *C. difficile* infection. Although no typing investigations were requested by Maidstone during the outbreak period, isolates from the hospital were examined under the DH/HPA surveillance programme in 2005–06 and this revealed that nine out of 10 isolates submitted belonged to type 027.

Research

So, what are the properties that make *C. difficile* such a successful microbe? Such is the current high profile of *C. difficile* that academics (and funding bodies) have realised that there is an urgent need to study this germ, and many studies are underway to try to understand its properties. Already, certain groups have discovered that the *C. difficile* genome is highly capable of acquiring extragenetic material that will be of selective advantage (e.g., mobile antibiotic resistance genes).[28]

In addition, a key factor in its spread and survival is its ability to produce spores (Fig. 1). These are shed into the environment during an episode of diarrhoea and can survive indefinitely until they are ingested by a susceptible host. Some studies have shown that certain strains produce more spores than others – an obvious survival advantage[29] – and it is important here to remind those in infection control that alcohol hand-rubs have no effect whatsoever on *C. difficile* spores.

The DH-HPA surveillance programme has revealed that common UK PCR ribotypes (i.e., types 027, 106 and 001) appear to be more resistant to certain antibiotics (e.g., the macrolides and fluoroquinolones) than are the less-common strains, and that there is widespread resistance to certain carbapenems across all strains. This suggests that antibiotic selection may play a role in which strains populate our hospitals, as bacterial resistance genes are only maintained in a population if they are of some use.

Summary

Much progress has been made in our understanding of the extent and epidemiology of *C. difficile* disease in the UK. However, there is still much to do in order to reduce this huge burden on our healthcare systems. New strains can arise with seemingly dramatic consequences and hospitals can find themselves overwhelmed with the problem.

Thanks largely to the media, such has been the rise in profile of *C. difficile* infections that politicians and the general public are now far more aware of the infections caused by this bacterium. There is even a *C. difficile* support website (www.cdiff-support.co.uk) at which sufferers and their relatives can obtain information and swap experiences.

No longer in obscurity, *C. difficile* has transcended the confines of the purely scientific literature and has taken its place alongside other modern-day hospital superbugs that are well and truly in the public domain. □

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