EPIDEMIOLOGICAL TYPING

PCR-ribotyping and pyrolysis mass spectrometry fingerprinting of environmental and hospital isolates of *Clostridium difficile*

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The relationships between environmental isolates of *Clostridium difficile* were examined by two typing methods, PCR ribotyping and pyrolysis mass spectrometry (PyMS). The 184 isolates were divided into 23 different PCR ribotypes, 13 of which were producers of toxins A and B; the remaining 10 types did not produce either toxin A or B. PyMS analysis resolved 31 groups with 60 (32.5%) isolates in one group (group 9). In both methods most of the isolates showed similar clustering. PCR ribotypes of the environmental isolates were compared with those of clinical isolates that had been typed previously. Seventeen PCR types (13 toxigenic PCR types and four non-toxigenic types) were found in both sets of isolates.

Introduction

Clostridium difficile is the causative agent of pseudo-membranous colitis (PMC) and most cases of antibiotic-associated diarrhoea [1, 2]. Outbreaks of *C. difficile* cross-infection may occur in elderly hospitalised patients and in care facilities for the elderly. Several different typing schemes have been developed for *C. difficile*, including unmodified SDS-PAGE [3], SDS-PAGE of ³⁵S-methionine-labelled cultures [4], bacteriocin-bacteriophage typing [5], restriction endonuclease digest fragment length polymorphisms [6], serotyping [7], immunoblotting of outer-membrane proteins [8], antimicrobial resistance patterns [9], pyrolysis mass spectrometry (PyMS) [10, 11] and polymerase chain reaction (PCR) ribotyping of the intergenic space between the 16S and 23S rRNA genes of the ribosomal RNA gene complex [12, 13]. Some of these methods are complex, expensive, limited to a few specialised laboratories, have poor discrimination and reproducibility, and may not type all strains of *C. difficile*.

PyMS typing for *C. difficile* has the advantage of rapid high throughput of strains with good discrimination. The system has the disadvantage that results cannot be compared from batch to batch and it does not assign a permanent type which can be useful in

the long-term epidemiological study of the species. PCR ribotyping is a new typing system which has been applied to various micro-organisms, and was first applied to *C. difficile* by Gurtler [12]. A modified method for routine typing of *C. difficile* was described recently [13]. This gives a permanent type designation for each strain, allowing results to be compared easily.

Recently a study examining the environment as a potential source of community-acquired human infections was conducted [14]. *C. difficile* was isolated from 184 of 2580 samples taken from farm animals, household pets, hospitals, family houses, student hostels, nursing homes for the elderly, soil and water. The majority (75%) of environmental isolates produced toxins A and B. To examine links between these 184 environmental isolates and clinical isolates from cases of PMC and AAD, the isolates were typed by PyMS and a modified PCR-ribotyping method.

Materials and methods

Bacterial strains

One hundred and eighty-four *C. difficile* isolates obtained from animals (cats, dogs, horses, pigeons, sheep) and from different environmental sites including soil, treated and untreated sources of water, hospital wards, family houses, nursing homes for the elderly and student hostels were examined [14]. The identity of the isolates was confirmed as described previously [15] and they were tested for the production of toxin A

(enterotoxin) and toxin B (cytotoxin) [15]. To compare the strains found in the general environment with those causing infection, 541 *C. difficile* isolates of clinical origin submitted to the Anaerobe Reference Unit (ARU; Cardiff) for the investigation of putative hospital outbreaks of AAD and PMC were included in the study. These isolates originated from hospitals throughout England and Wales.

Pyrolysis mass spectrometry

One colony was subcultured from each isolate on to Fastidious Anaerobe Agar (FAA; Lab M, Bury) and incubated anaerobically (Concept 300 Anaerobic Workstations; Fred Baker Scientific Ltd, Bradford) in an atmosphere of N₂ 80%, CO₂ 10%, H₂ 10% for 48 h at 37°C. The growth was sampled with a straight wire and transferred to each of four replicate pyrolysis foils which were heated to 100°C for 10 min to inactivate enzymes and dehydrate the specimen within 10 min of sampling. Foils were then stored in a vacuum desiccator before automated processing on a PMS 200X (Horizon Instruments, Heathfield, Sussex) pyrolysis mass spectrometer with pyrolysis for 4 s at 530°C and analysis of the pyrolysis products by mass spectrometry. Data were analysed as described previously [16].

PCR ribotyping

This was performed by the method of O'Neill *et al.* [13]. Single colony subcultures were grown anaerobically at 37°C for 24 h on FAA in an atmosphere of N₂ 80%, CO₂ 10%, H₂ 10%. DNA was extracted from a suspension of 10–12 colonies in 100 µl of Chelex 100 (BioRad Laboratories, Hercules, CA, USA) 5% by heating for 10 min at 100°C in a waterbath. PCR was performed with the primers P3 (5'-CTG GGG TGA AGT CGT AA CAA GG-3') and P4 (5'-GCG CCC TTT GTA GCT TGA CC-3'). Amplifications were performed in a final volume of 100 µl with a reaction mixture containing 1.5 mM MgCl₂, 10 mM Tris HCl (pH 9), 50 mM KCl, Triton X-100 0.1%, 2.5 U of Taq polymerase (Promega, Madison, WI, USA), 200 µM of each dNTP, 50 pmol of each primer and 10 µl of template DNA preparation.

Amplification consisted of 35 cycles of denaturation at 96°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 2 min. A negative control consisting of reaction buffer, primers, dNTPs, Taq polymerase and distilled water was included with each PCR run. The PCR products were separated electrophoretically on Metaphor Agarose (FMC BioProducts, Rockland, ME, USA) 3% at 150 V for 6 h. DNA fragments were visualised by staining with ethidium bromide 0.5 mg/L. Gels were photographed under UV illumination and the data were saved to floppy disc for analysis by the Gel Compar software (Applied Maths, Belgium). Isolates were compared by using the

Jaccard coefficient with band tolerance set at 2.5%. Dendrograms were generated by unweighted pair group mean association (UPGMA). Isolates were assigned to different PCR types if they differed by a single band. Representative patterns of the different PCR ribotypes identified among 184 environmental isolates of *C. difficile* were examined three times to assess the reproducibility of the method.

Results

Results of PyMS fingerprinting of the environmental isolates showed one major group (group 9) comprising five subgroups containing 69 (37%) of the 184 isolates. The remaining isolates were distributed among 30 other groups. Twenty-three distinct and reproducible banding patterns were found by PCR ribotyping. The number of bands per strain was in the range five to nine with an average of seven, in the size range 257–601 bp (Fig. 1). Thirteen PCR types were uniformly positive for toxins A and B, and 10 were uniformly negative for both toxins (Fig. 2). The correlation between PCR ribotyping and PyMS fingerprints is shown in Fig. 3.

PCR type 1 was the largest cluster comprising 76 (41%) of the 184 isolates. PCR type 1 contained 58 (76.3%) of the hospital isolates demonstrating significant contamination of the hospital environment by this particular type. Eighteen other isolates belonging to PCR type 1 were found also in soil (1), water (7), animals (1), family houses (2), student hostels (3), and from nursing homes for the elderly (4). Fifteen further PCR-types each contained between two and 13 isolates, and the remaining seven PCR types each contained a single isolate. Some PCR types comprised mostly isolates from one source, e.g., types 2, 5 and 34 (water), type 9 (soil) and type 33 (veterinary clinic), whereas other PCR types containing more than one isolate contained isolates from several different sources. The 23 PCR ribotypes of environmental isolates were compared with the 64 PCR ribotypes found in the library of 541 clinical isolates of *C. difficile*; 17 PCR ribotype patterns found in clinical strains matched those in environmental sources. There were six PCR ribotypes found only in environmental isolates and the remaining 41 PCR ribotypes were found only in clinical isolates.

Discussion

Several methods have been employed to characterise and distinguish between different strains of *C. difficile*. The majority of typing methods have been aimed at investigating putative common sources of outbreaks in cross-infection incidents in hospitals. However, typing schemes have so far contributed little to the overall understanding of the extra-nosocomial epidemiology

Fig. 1. Representative patterns of the different PCR ribotypes identified among 184 environmental isolates of *C. difficile*. (a) Lane 1, 100-bp size marker; 2, PCR type 6; 3, type 34; 4, type 2; 5, type 42; 6, 100-bp size marker; 7, type 11; 8, type 14; 9, type 5; 10, type 8; 11, 100-bp size marker. (b) Lane 1, 100-bp size marker; 2, type 35; 3, type 26; 4, type 10; 5, type 41; 6, 100-bp size marker; 7, type 40; 8, type 9; 9, type 38; 10, type 30; 11, 100-bp size marker. (c) Lane 1, 100-bp size marker; 2, type 1; 3, type 46; 4, type 23; 5, type 44; 6, 100-bp size marker; 7, type 12; 8, type 39; 9, type 33; 10, 100-bp size marker.

Fig. 2. PCR ribotyping of animal and environmental isolates. LH, Llandough Hospital; UHW, University Hospital of Wales; CRI, Cardiff Royal Infirmary.

Fig. 3. Grouping of *C. difficile* isolates by PyMS and comparison with PCR-types.

and ecology of *C. difficile*. In this study, PyMS was used to demonstrate the great diversity of strains obtained from a wide range of different environmental sites. There was a close relationship between the hospital environmental isolates, notably in PyMS group 9, indicating the high prevalence of this type in the hospital environment. PCR ribotyping was used to allocate a definite type designation to each *C. difficile* isolate, allowing results to be compared easily. All isolates in PyMS group 9 corresponded to isolates belonging to PCR type 1. PyMS subdivided some PCR-types, but broadly confirmed the PCR results. PCR ribotyping is a relatively new typing scheme which has been applied to a variety of organisms including *Escherichia coli* [17], *Burkholderia cepacia* [18] and *Staphylococcus aureus* [19].

Of the environmental isolates, 139 (75.5%) produced toxins A and B and so were theoretically capable of inducing disease in susceptible patients. The isolates found in the hospital environment tended to be indistinguishable, e.g., PCR type 1. In many hospital settings there is often one endemic or even epidemic strain while in others a variety of strains may be responsible for outbreaks of *C. difficile* infection. The PCR ribotyping results were in agreement with this interpretation. With isolates from four hospitals in one district, 56 (89%) of the 63 isolates from three hospitals (Cardiff Royal Infirmary, University Hospital of Wales and Caerphilly District Miner's Hospital) belonged to PCR type 1, whereas the 13 isolates from Llandough Hospital were divided into five PCR ribotypes with just two isolates belonging to PCR type 1. Isolates from other sources such as water or soil showed greater dissimilarity. Nosocomial acquisition of *C. difficile* is well documented, but it is not yet clear how *C. difficile* is acquired in the community. A link between environmental strains and clinical strains responsible for cases of AAD and PMC is suggested for some PCR types found in both the environmental sets and strains of clinical origin typed at the ARU in Cardiff. Of the 23 environmental PCR ribotypes, 17 were matched with similar PCR ribotypes found in clinical strains. However, there was greater diversity of PCR ribotypes and PyMS types among environmental strains than was found in routine typing of clinical isolates. The matching PCR ribotypes were from different animals and environmental sites indicating possibilities of environmental acquisition of *C. difficile*. This supports the findings of O'Neill *et al.* [20] who reported that apparent relapses of *C. difficile* infection were either re-infection with the same strain or another which may have been acquired from the environment. Overall, typing clearly showed that similar PCR-types are found in both clinical and environmental isolates in the UK demonstrating the

potential importance of the environment as a source of *C. difficile* infection in man.

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