1. SUMMARY

A 1947 base pair (bp) fragment of the toxin A gene of Clostridium difficile was sequenced. A continuous open reading frame was found, which contained 4 distinct groups of repeat nucleotide sequence with 88 to 100% identity within each group. The arrangement of the groups (A, 81 bp, B, C and D, 63 bp) was ABCCDABCDDABCDABCCDABCDABCCDABCDABC. Based on nucleotide sequence data from the C repeat group, a pair of oligonucleotide primers were synthesised and used in the polymerase chain reaction (PCR) to amplify fragments from the toxin A gene. Several products of multiples of 63 bp length were amplified for all 33 toxigenic C. difficile strains tested in contrast to the 12 non-toxigenic strains tested which failed to amplify any product. This rapid technique is of potential use in the specific identification of toxigenic C. difficile strains in mixed culture and from clinical specimens.

2. INTRODUCTION

Clostridium difficile is the aetiological agent of pseudomembranous colitis and antibiotic-associated diarrhoea in humans. The pathogenicity of C. difficile is, in part, related to the production of at least two toxins, toxin A, an enterotoxin, and toxin B, a cytopathic toxin [1,2]. Currently, diagnosis depends on the isolation and identification of the organism, which takes up to 48 h, and/or the demonstration of toxins in faecal specimens of patients using tissue culture or ELISA techniques. More rapid diagnosis is essential to enable prompt treatment.

We have undertaken the cloning and sequencing of the toxin A and B genes to develop improved methods for the specific identification of toxigenic strains and to gain an insight into the molecular basis of C. difficile-related disease. Previously, we described the clone λA5 with a 14.3 kb insert which encodes the toxin A gene [3]. The protein product of at least 235 kDa expressed by λA5 reacted with antiserum to the purified toxin, agglutinated rabbit erythrocytes and had a cytopathic effect on Chinese hamster ovary cells, properties consistent with purified toxin A protein. A 4.5 kb PstI subclone, designated pBWW47, was isolated, which expressed a 140 kDa protein that...
retained haemagglutinating capacity but was no longer cytopathic. Sequencing of this fragment revealed 4 distinct groups of repeat nucleotide sequence.

Recently, the polymerase chain reaction (PCR) has been shown to be useful for the detection of slow-growing organisms such as *Mycobacterium tuberculosis* [4,5] and *Helicobacter pylori* [6]. PCR requires nucleotide sequence data to design oligonucleotide primers for the polymerisation step. The aim of this study was to utilise nucleotide sequence data from tandemly arranged repeat groups to design suitable oligonucleotide primers for the PCR amplification of the toxin A gene, to aid in the development of a rapid, sensitive and specific method for the identification of toxigenic *C. difficile* strains.

3. MATERIALS AND METHODS

3.1. Isolation of DNA and restriction enzyme analysis

DNA from clone λT5 containing the toxin A gene from *C. difficile* strain W1, was isolated using the plate lysate method [7]. DNA from the plasmid clone pBWW47 was isolated by alkaline lysis [8]. Both clones were mapped with a variety of restriction enzymes (Amersham International, Amersham, U.K.) by established procedures [7].

3.2. M13 cloning and DNA sequencing procedures

DNA fragments within pBWW47 were subcloned in both orientations into M13 and both strands were sequenced by the dideoxy chain termination procedure [9]. Extended polyacrylamide gel runs were performed to confirm the arrangement of overlapping repeat groups of nucleotide sequence.

3.3. Bacterial strains

Nine standard *C. difficile* strains from different typing groups [10,11] designated A to E and W to Z, were used for initial PCR experiments. Strains B, D, E, W, X and Z were previously shown to be toxigenic by a direct sandwich ELISA using antitoxin A [12] in contrast to the non-toxigenic strains A, C and Y. Thirty six other *C. difficile* strains isolated from patients attending St. Bartholomew's Hospital including 9 non-toxigenic strains were tested in PCR experiments. Strains were grown anaerobically at 37°C for 36 h on selective media (cycloserine, cefoxitin and fructose agar, Oxoid Ltd., Basingstoke, U.K.) and their identity confirmed by colonial morphology, smell, Gram stain and gas-liquid chromatographic analysis of volatile fatty acids [13]. Sixteen other clostridial strains [14] from 12 other species including 3 *C. sordellii* strains were also studied by PCR analysis.

3.4. Synthetic oligonucleotides

All oligonucleotides were synthesised on an Applied Biosystems synthesiser with the automated phosphoramidite coupling method. Oligonucleotides used as primers were designed based on nucleotide sequence data from repeat group C (BW 69 5'-GAAGCAGCTACTGGATGGCA and BW 70 5'-AGCAGTGTTAGTATTAAAGT).

3.5. Preparation of samples for PCR and PCR methodology

A single colony was scraped with aq inoculating loop into a 1.5 ml polypropylene tube containing 200 μl of sterile water. After boiling for 10 min the samples were spun at 14000 × g in a benchtop microfuge for 5 min. Two microlitres of supernatant liquid were added to a 100 μl reaction volume with 1.5 mM magnesium chloride, 10 mM tris-HCl (pH 8.3), 0.01% gelatin, 200 μM each of deoxyribonucleotides, 100 μM oligonucleotide primers and 2.5 U *Taq* polymerase (Perkin Elmer Cetus, California U.S.A.). Reaction mixtures were overlaid with paraffin oil (100 μl), placed in a thermal cycler (Hybaid Ltd, Twickenham, Middlesex, U.K.) and amplified for 30 cycles. A rapid two step cycle of 94°C and 46°C for 30 s each was chosen with the first 94°C step extended to 3 min to ensure denaturation of the initial sample. Twenty microlitres of the amplified products were electrophoresed in a horizontal 4% NuSieve GTG (ICN Biomedicals Ltd, High Wycombe, U.K.) agarose gel containing 0.5 μg ethidium bromide per ml and the bands were visualised by excitation under ultraviolet light.
To determine the sensitivity of the PCR in detecting toxigenic *C. difficile* strains in the presence of bacteria from a faecal specimen, the number of organisms present in a PCR sample was quantified by serial dilutions and a viable count on Columbia horse blood agar plates (Oxoid Ltd.).

4. RESULTS

DNA isolated from the clones λA5 and pBW47 was mapped with a variety of restriction enzymes shown in Fig. 1. Sequencing of a 1947 bp internal fragment of the toxin A gene revealed the presence of 4 distinct groups of repeat nucleotide sequence (A, 81 bp, B, C and D, 63 bp) with 88 to 100% identity within groups. The arrangement of the groups which accounts for 1935 of the 1947 bp sequenced is shown in Fig. 2, and the other 12 bp were 2 long areas of 6 bp between the underlined A and D groups. Also shown in Fig. 2 is the consensus nucleotide sequence and translated peptide sequence for groups A, B, C and D. The 4 groups showed areas of similarity to each other, in particular, the central region of the B and C groups. Translation of the entire 1947 bp sequence (EMBL data bank accession No X17194) revealed a continuous open reading frame (orf) confirming that the DNA encodes an internal region of a large polypeptide.

From nucleotide sequence data oligonucleotide primers BW 69 and 70 were synthesised from the tandemly repeated C group. Using these primers in the PCR with toxigenic strains B, D, E, W, X and Z, revealed DNA bands of 63, 126, 189, 252 and 333 bp length which were absent from non-toxigenic strains, A, C and Y (Fig. 3). A total of 45 *C. difficile* strains were tested in the PCR and all 33 toxigenic strains were positive in contrast to the 12 non-toxigenic strains. Of the other 16

(ii)

\[ \text{ABCCCDABCCDABCCDABCCDABCCDABCD} \]

(i)

\[ \text{ABCCCDABCCDABCCDABCCDABCCDABCD} \]

Fig. 2. (i) Arrangement of nucleotide repeat groups A, B, C and D. (ii) Consensus nucleotide and peptide sequence for repeat groups A, B, C and D.
clostridial strains from 12 different species tested, 3 C. sordellii strains were positive (Table 1).

To determine the number of bacteria necessary to give visible bands on agarose gels after PCR, samples containing known numbers of bacteria from viable counts were serially diluted and subjected to amplification. Visible bands were seen with as few as 300 (lane 1, Fig. 3) and 30 (lane 2, Fig. 3) toxigenic bacteria even in the presence of $10^7$ anaerobic enteric bacteria.

5. DISCUSSION

This study has revealed within the toxin A gene the presence of several repeat groups of nucleotide sequence which translate into groups of peptide sequence similar to those reported by Johnson et al. [15] for C. difficile strain VPI 10463. The expressed product of clone pBWW47 which includes the area of DNA sequenced retained the ability to agglutinate rabbit erythrocytes (unpublished results), thus the peptide portion of the toxin A gene encoded by the repeat sequence may play a role in the specificity of the toxin protein to bind to receptor molecules on the brush border.

Table 1

Summary of PCR experiments with various clostridial strains

<table>
<thead>
<tr>
<th></th>
<th>No. tested</th>
<th>PCR positive</th>
<th>PCR negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxigenic C. difficile</td>
<td>33</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>Non-toxigenic C. difficile</td>
<td>12</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Other Clostridial species</td>
<td>16</td>
<td>3</td>
<td>13</td>
</tr>
</tbody>
</table>

Fig. 3. PCR of toxigenic and non-toxigenic C. difficile strains using oligonucleotide primers BW 69 and 70: Lanes B, D, E, W, X, and Z, toxigenic C. difficile strains from pure colonies, lanes A, C and Y non-toxigenic C. difficile strains from pure colonies, lanes 1, 2 and 3 serial dilution of C. difficile strain W with 300, 30 and 3 organisms respectively mixed with $10^7$ other anaerobic enteric bacteria.

Lane M: DNA size marker of visible bands, 67, 80, 110, 147, 190, 242 and 267 bp (Bsh1 and Msp1 digests of pHC314).
membrane of the human intestine. Interestingly, a haemagglutinin from Myxococcus xanthus has been shown to contain 4 highly conserved repeat groups of 67 amino acid residues [16].

The presence of at least 11 C repeat groups of nucleotide sequence may have heightened the sensitivity of the PCR which could specifically detect the presence of as few as 30 toxin-producing bacteria in the presence of $10^7$ other bacteria. This sensitivity is approximately 3 times greater than that reported by Karch and Meyer [17] for the detection of Shiga-like toxin-producing E. coli strains using PCR methodology. The PCR products of 63, 126, and 189 bp seen in Fig. 3 can be explained by the amplification of DNA between the 3 adjacent C groups. However, amplification of the fourth 252 bp band, which was clearly observed for all toxigenic strains tested, is difficult to explain from the arrangement of the repeat groups as 4 adjacent C groups or 2 C groups separated by 2 other 63 bp groups are not apparent. It is possible that such an arrangement of repeat groups exists outside the area of sequence reported in this study. The presence of the fifth band of approximately 333 bp can be explained by the 2 C groups separated by D, A and B groups. The profiles from the PCR products appeared identical for all 35 toxigenic strains tested which were from a variety of typing groups suggesting that the area of DNA to which the primers annealed are highly conserved between different toxigenic strains. The variation in PCR conditions and the use of new pairs and various combinations of primers may reveal further banding patterns which apart from their diagnostic potential should prove useful in typing different C. difficile strains.

The failure to detect DNA amplification products from all 12 non-toxigenic strains tested can be explained by the absence of at least part of the toxin A gene. These observations have been confirmed by hybridisation studies using the 4.5 kb PstI fragment as a toxin A gene-specific probe [18]. The positive PCR results obtained with the C. sordellii strains were not surprising as the organism has a haemorrhagic toxin which cross-reacts immunologically [19] and shows DNA homology with the toxin A gene [18,20].

The PCR experiments in this study were designed for the rapid amplification of target DNA. The $72^\circ C$ elongation step normally incorporated in the PCR was omitted as the relatively small length of DNA required to be synthesised could be completed by the Taq polymerase as the $46^\circ C$ to $94^\circ C$ cycle transiently passes the optimum temperature for polymerisation. The 10 min boiling of the samples released enough bacterial DNA for the PCR to yield positive results. This rapid and convenient method requires no DNA extraction procedure in contrast to that reported for the PCR on mycobacteria [5].

The PCR-based method described in this study, which does not require hybridisation technology or the use of radioactivity, should be useful for the rapid identification of toxigenic C. difficile strains from pure and mixed culture and, with suitable adaptations, could be applied directly to clinical specimens.

ACKNOWLEDGEMENTS

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REFERENCES