RESTRICTION MAPPING OF TOXIN A GENE FRAGMENT IN CLOSTRIDIUM DIFFICILE

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ABSTRACT

Toxin A variant strain was identified by restriction endonuclease analysis (REA) as type CF2, was not detected by an immunoassay for C. difficile toxin A. Culture supernatants of CF2 failed to elicit significant enterotoxic activity in the rabbit ileal loop assay but did produce a typical cytopathic effects in cell culture assay. Southern hybridization, PCR amplification, and DNA sequence analysis were performed on the toxin A (tcdA) and toxin B (tcdB) genes of type CF2 isolate. According to these facts we had tried to obtain new restriction map for the fragment of these genes by PCR and (restriction fragment length polymorphisms) - RFLPs methods using various restriction enzymes such as Hind III, pstI and Eco RI. In this work various fragments had been identified with 250-700bp lengths. Restriction map of fragment 250bp was preformed. The results showed a new restriction map by which obtained a new variation at the same fragment.

Key words: Clostredium difficile, Toxin.
الخلاصة

شُكُّدت السلالات (العُزلات) البكتيرية C. difficile من النوع CF2 إلى النوع A و B إلى النوع B، و A إلى النوع C. difficile المتغيرة والمنتجة للسموم طرق حديثة مثل تحليل خارطة القطع كوسيلة بديلة عن الطرق المناعية المتبعّة سابقاً، أو باستخدام طرق التفاعل التنسلسي للبوليميرز PCR و طريقة Southern hybridization وكذلك طرق تحليل A لغرض تحليل بنية الجين التسلسل الدنا للذات CBF2. أما بالنسبة للفترة االجينا A (tcdA) و B (tcdB) والجين B (tcdA) والجين B (tcdB) لغرض تحليل بنية الجين تسلسل الدنا للفترة االجينا A و B. وعلى التوالي من السلالات المختلفة، وعلى ضوء هذه المعطيات كان البحث منصباً على إيجاد خارطة قطع لقطعة محددة ضمن الجين A ومقارنتها بما تم الحصول عليه عالمياً، باستخدام PCR كطريقة لترميم المستخلص من عزلات مرضية مختلفة. استخدمت لهذا الهدف انزمات قطع مختلفة: DNA DNA EcoRI و Hind III و PstI لغرض تحديد خارطة القطع للقطع المستهدفة. وتتم تحديد حجوم متباعدة من تتراوح بين 250-700bp. تشير نتائج البحث بأنه تم الحصول على خارطة قطع جديدة بالمقارنة مع المعطيات المائلة والتي من خلالها تم التعرف على تغيرات واضحة ضمن الجين A. وبحلول القطعة المستهدفة.
INTRODUCTION
The disease pseudomembrane colitis (PMC) has been recognized for quite sometime. One of the earliest descriptions of the disease was reported in 1893 by Finney(1), who noted pseudomembranes and hemorrhagic diarrhea in a young woman following surgery. The number of reported cases of PMC increased dramatically, however, following the widespread use of broad-spectrum antibiotics.

The molecular analysis of Clostridium difficile toxins started in the 1980s with the first attempts to clone fragments of toxin genes and has progressed with the sequencing of both toxin genes(2,3,4,5), with the definition of the region encoding the toxins, and with studies of toxin gene regulation(6,7,8). Unfortunately, in parallel with developments on the molecular biology and biochemistry of C. difficile, several different nomenclature systems have been applied to the toxins and their associated genes. With an increasing number of research groups working on the molecular biology of C. difficile, or using its toxins as tools in cell biology, and with the imminent finalization of the first C. difficile genome sequence, the need for a unified nomenclature has become apparent. A similar approach was taken on naming the clostridial neurotoxins, where a proposed unified nomenclature (8) has resulted in consistent.

TcdA and TcdB are both encoded on the same 19.6-kb pathogenicity locus (Fig. 1) in C. difficile(9). The two toxin genes are closely situated, with a 1,350-nucleotide intervening sequence on this locus, and are transcribed in the same direction. In addition to tcdA and tcdB, three other open reading frames are located on this pathogenicity locus and are thought to be involved in regulation.

Fig. (1): Genetic arrangement of the Clostridium difficile pathogenicity locus and proposed protein domain structures of TcdA and TcdB. From Voth and Ballard (11).

Department of Biology, College of Science, Basrah University of toxin production or release of the toxins from the cell(9). tcdC lies downstream of tcdA and is transcribed in the opposite direction from the two toxin genes, and tcdC is highly expressed in early exponential phase but declines as growth moves into the stationary phase. This decline in TcdC expression corresponds to increases in TcdA and TcdB, suggesting that TcdC
may function as a negative regulator of toxin production. \textit{tcdD} is found upstream of \textit{tcdB} and is coordinately expressed with both of the toxin genes. \textit{TcdD} is similar to DNA-binding proteins and has been shown experimentally to enhance expression of promoter reporter fusions containing the promoter-binding regions of \textit{tcdA} and \textit{tcdB}. \textit{TcdD} is also homologous to \textit{TetR} and \textit{BotR}, which serve as positive regulators of tetanus and botulimum toxin synthesis, respectively (10,11).

A restriction map is derived by determining the location of a number of restriction endonuclease cleavage sites on the DNA relative to each other. Partial restriction map of this 15-kb region of the \textit{C. difficile} genome is shown in Fig.(2). Clone pCD11 has been partially characterized and shown to contain a carbohydrate binding region and antigenic epitopes which react with the monoclonal antibody PCG-4 (36). Clone pCD11R-6, in addition to containing the entire pCD11 insert and most of the pCD11L insert, contains the last 80 bases of the toxin A gene and approximately 4.1 kb of additional sequences downstream from the toxin Agene. The downstream region contains two open reading frames (ORFs) and part of the third. All of these ORFs read in the direction opposite that of the toxin A gene (data not shown). Clone pCD11L contains an additional 1.5 kb of sequence upstream of the pCD11 insert. Clone pCD17 was used as a probe for cloning pCD19. Clone pCD19 codes for the 5' end of toxin A, a small ORF that could code for a 16- or 19-kilodalton (kDa) protein and 1.2 kb of toxin B.

According to the endonuclease restriction map of the toxin A gene of \textit{C. difficile} strain VPI 10463 (4), the amplified 546-bp DNA sequence was encompassed in a 5.3-kbp \textit{HindIII}-digested DNA fragment, and the amplified 1,266-bp DNA was encompassed in a 9.7-kbp \textit{HindIII} digested DNA fragment. As expected, the 546-bp probe hybridized with a single 5.3-kbp fragment Fig.(2).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Partial restriction map of toxin A gene of \textit{Clostridium difficile}. From Dove et al. (4).
}
\end{figure}

For construction of the toxin A gene, three restriction fragments encompassing the \textit{C. difficile} toxin A gene and a smaller ORF a short distance downstream from the toxin gene were ligated to produce the 9.4-kb construct pCDtoxA.6. Exonuclease digestion removed all but 33 bases of the ORF located at the 3' end of the toxin A gene, resulting in a clone designated pCDtoxA.03. Digestion with restriction endonucleases indicated that the gene fragments were properly inserted in the 8.6-kb insert of pCDtoxA.03 (data not shown). In addition, sequencing across the construct junction sites confirmed the
correct ligation of the gene fragments(12). Transposon (Tn1000) mutagenesis. The 0.27-kilobase fragment and the 1.9-kilobase insert were radiolabeled and used as DNA probes in hybridization studies. Southern blot analysis with the gene probes against chromosomal DNA from Cmr strains of *C. difficile* obtained from five distinct geographical locations revealed that at least two copies of the same chloramphenicol acetyltransferase gene were present for each strain. Hybridization of the gene probes against Cmr strains of *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Klebsiella edwardsii*, *Escherichia coli*, and to four other clostridial species revealed no homology even under conditions of low stringency (11).

Sambol. et al. (22) cloned fragments of toxin B (*tcdB*) and toxin A (*tcdA*) into PCR-Script Amp vectors (Stratagene) and transformed into *Escherichia coli* strain XL-10 Blue (Stratagene) with the PCR-Script Amp cloning system (Stratagene) according to the manufacturer’s instructions genes, used for DNA sequencing of *C. difficile* variant type CF2 isolate 5340. The locations of the primers used to amplify gene fragments from *C. difficile* variant type CF2 are shown in Fig.(3).

![Fig.(3) Cloned fragments of toxin B (*tcdB*) and toxin A (*tcdA*) genes used for. From Sambol et al. (2).](image)

Ackerman *et al.* (13), amplified the entire *tcdA* gene of *C. difficile* with the subsequent cloning and expression of the gene in competent *E. coli* by means of an expression vector.

A toxinotype was characterized by similar patterns of changes in the toxin genes and in other regions of the pathogenicity locus and also similar pulsed-field gel electrophoresis patterns. Parts of the toxin genes were amplified, and the PCR fragments were checked for length polymorphisms and cut with several restriction enzymes to monitor restriction fragment length polymorphisms (RFLPs). Polymorphisms were usually observed in both toxin genes. Restriction sites seemed to be less heterogeneous in the *tcdA* gene, in which for most enzymes only two different RFLPs were recognized. However, deletions were observed in *tcdA*, and four new types of shortened *tcdA* genes are described. Strain SUC 36 (toxinotype XVI), on the other hand, was similar to well-defined group consisting of toxinotypes V, VI, and VII, which thus far includes only A-B+. Formerly described variant strains of serogroup F belonged to toxinotype VIII, and isolate 8864 belonged to toxinotype X. For the majority of
toxinotypes, more than one isolate was found. All toxinotypes except types I and II had changes in both the \textit{tcdB} and the \textit{tcdA} toxin genes. Toxinotypes IV to X had an insertion upstream of \textit{tcdD}, and for toxinotype X another insertion upstream of \textit{tcdA} was characteristic Fig.(4). The same \textit{tcdB} type was observed in conjunction with different \textit{tcdA} genes, like in toxinotypes V, VI, and VII or 0, I, and II. Toxinotypes IX and X had very similar \textit{tcdB} genes as well. On the other hand, the identical type of \textit{tcdA} gene was found in toxinotypes IX and III, together with different \textit{tcdB} genes. These similarities among toxin genes or domains in various toxinotypes probably reflect phylogenetic differences or relationships (14,15).

![Fig.(4)]: A schematic representation of the PaLoc region with newly described toxin types. From Geric \textit{et al.} (14).

**MATERIAL AND METHODS**

**Isolation and Identification of \textit{Clostridium difficile} from stool specimens**

Stool specimens were collected and frozen at \(-20^\circ\text{C}\) within a few hours until use. \textit{C. difficile} spores were isolated by homogenizing equal amounts of specimen and ethanol (approximately 1ml of each) are mixed and incubated for 30min at room temperature. One drop (approximately 0.1ml) of the treated specimen is plated onto anaerobic blood agar and Cycloserine-cefoxitin-fructose agar (CCFA) plates were streaked for isolation and incubated an aerobically at 37°C for 48hrs. Remainder of specimens were frozen immediately after processing at or below \(-20^\circ\text{C}\) for later use. The isolates were identified as \textit{C. difficile} by their unique odor and colonial morphology. Gram’s stain and fluorescence under long-wave ultraviolet light, API 20A (Bio-Merieux, Marcy I Etoile, France) are also employed.

**DNA extraction**

Genomic DNA was isolates by Kato \textit{et al.}(16). For the preparation of genomic DNA, a single colony was suspended in 50ml of TES. The suspension was heated at 95°C for 10min. and centrifuged at 15,000x \textit{g} for 2min. The suspension was transferred to 1.5ml Eppendorf tube and stored in the freezer at \(-20^\circ\text{C}\) for later use. For PCR assay one microliter of the resultant suspernatant was added to 50µl of reaction buffer consist of 25µl of PCR mixture, 1µl of each primer, 1µl template DNA and 22µl D.W. One primer was used to detect toxin A gene NK2-NK3 were derived from the non-repeating of \textit{C.
difficile toxin A gene. The thermal profile for primer was 35 cycles comprising 95°C for 20 s and 55°C for 120 s.

The restriction map

Three techniques are central to the mapping of N2-N3 fragments of DNA: The first technique restriction endonucleases digest, the second digest with different restriction enzyme, the third technique is use of electrophoresis to separate DNA fragments according to the size.

The following to 1.5ml microcentrifuge was made. All volumes are in micro liter (µl), and the reaction volume is 20µl.

<table>
<thead>
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<th>Tube</th>
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<td>Hind III (10U/µl)</td>
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<td>Pst I  (10U/µl)</td>
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RESULTS AND DISCUSSION

When the N2-N3 fragments are digested by the restriction endonuclease, EcoR1 two fragments, sized 105 and 145bp are produced. When digested by a second restriction endonuclease, HindIII two fragments are also produced but these are 50 and 200bp in size. The single digest with PstI generates fragments so it too has two target sites within the fragment these are 100 and 150bp in size. Digestion with mixture of two enzyme EcoR1+HindIII yield three fragments sized 145, 45 and 50bp. When digested with mixture of two enzyme PstI +HindIII yield three fragments 130, 70 and 30.

Also the EcoRI+PstI double digest generates only three fragments sized 85, 40 and 60bp. Since each of these enzymes has two target sites, it is to be expected that four fragments would be generated by the double digestion. There are two explanations for finding only three fragments.

1-One of the target sites for one of the two enzyme may lie very close to one of the target sites for the other enzyme. Upon digestion a very small fragment would be generated and such fragments are difficult to detect following electrophoretic separation.

2-The expected four fragments may be generated but two are, by chance, so nearly similar in size that they have not been separated by electrophoresis.

It is easy to distinguish between these explanations by considering the sum of the sizes from the double digest, which is 85+60+40=185bp. Since the N2-N3 fragment is 250bp, the fragments sized 65bp must have been generated by the double digest to account for the apparent shortfall; thus we need to reconcile the production of fragments sized 40, 60, 65 and 85bp with the map. We must concluded that there is no target site for PstI within the 65bp fragmented by EcoRI digestion, and that both PstI target sites
therefore lie within the part of the N2-N3 fragment between the two EcoRI sites which are separated by 250bp of fragment.

Double digestion with HindIII and PstI gives the three fragments; thus the target sites for the two enzymes are well separated and no pair of fragments are similar in size. To reconcile the products of double digestion with those from digestion with each enzyme singly. There is only one way that this is possible. The 200bp fragment given by HindIII digestion must give rise to the 20 and 130bp fragments from double digestion, i.e. both PstI target sites must lie within this 200bp sequence.

Also double digestion with HindIII and EcoRI gives the three fragments. Thus both HindIII sites occur within the 65 and 40bp sequences generated by EcoRI, bands of restriction fragment showing in Fig.(5).

Digestion with all three enzymes generates only three fragments sized 85, 45 and 60 bp, but we knew three should be six since we have already deduced that all the target sites are well separated. Also observe that the sum of the fragment sizes from triple digestion is 190 not 250bp as expected ,i.e. that 60bp of sequence is unaccounted for. The missing three fragments and the 60bp of DNA must be account for by the generation of fragments of nearly similar size, which are not separated by electrophoresis. The three extra fragments must be 10, 20, 30bp in size. The triple enzyme digestion therefore generates 85, 45, 60 and 10, 20, 30bp fragments. The complete map is therefore as shown in Fig.(6).

Phelps et al.(17) showed partial restriction endonuclease map of PCDtoxA.03 and recombinant clone fragments used for construction of the intact toxin A gene No restriction map of N2-N3 gene fragment. The clone with the smallest DNA insert size (pPPM9) was mapped with a variety of restriction endonucleases (AccI, AvaI, BamHI, BscI, DdeI, EcoRI, EcoRV, HincII, HindIII, MboI, PstI, SmaI, Stul, TaqI, XbaI, and XboI) as specified (11).

Several parameters influence the choice of an appropriate restriction enzyme. One of this length of recognition sequence :restriction enzymes with a 4-base recognition motif are generally preferable for DNA ,while 6-base cutters are more suitable for the less complex genomes of fungi (10)

The use of restriction endonuclease is facilitated by the available of a physical map of the DNA to be analysis. A physical map is derived by determining the location of a number of restriction endonuclease cleavage sites on the DNA relative to each other. The strategy followed for physical mapping depends largely on the size of the DNA and the number of cleavage sites of the used restriction endonuclease . Moreover , mapping of DNA fragments contained in a cloning vehicle is facilitated by the availability of detailed physical map of these plasmid.

For physical mapping it is necessary to determined the molecular weights of the cleavage products of the restriction endonuclease. If no cleavage sites are known on a plasmid, it best to start with two or more restriction endonuclease that cleave the DNA only a few times.

The position of some fragments can also be identified by examining genetic deletion mutants. If the deletion dose not eliminate a restriction site, a new fragment will appear in the digest of the deletion mutant and will be smaller, by the size of deleted DNA (18).
A physical map can then be deduced by comparing the cleavage patterns of single digestion with those of double digestions. Restriction map is beneficial in genetic engineering for:

1. Select suitable restrict enzymes to cut desirable gene.
2. Determine DNA sequences.
3. Early diagnostic for some genetic disease.

According to the previous studies, no restriction map of N2-N3 gene fragment, so we can consider these data is the first study.

![Image](image1.png)

**Fig.(5):** Restriction enzyme of N2-N3 gene fragment. M. 1.5kbp ladder marker; lane 1, *PstI*; lane 2 *HindIII*; lane 3, *EcoR1*; lane 4, *PstI+HindIII*; lane 5, *PstI+EcoR1*; lane 6, *EcoR1+HindIII*; lane 7, *HindIII+EcoR1+PstI*

![Image](image2.png)

**Fig.(6):** (a) Clarify restriction Map of N2-N3 gene Fragment 250 bp. (b) Linear map.
REFERENCES


