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Restriction Map For NK104-NK105 of Nonrepeating Region For *tcdB* Toxin Gene in *Clostridium difficile*

Thaer A. Ali Al-Edani [(1)]* , Jalal Y. Mustafa [(2)], Suhyila F. Ali [(1)]
[(1)] *Science College - Biology Department /Basra University* [(2)] *Veterinary Medicine College/Basra University*

Abstract: *This study was carried out for detected of the restriction map of tcdB gene for A^-B^+ isolates were isolated from patients with a colitis disease in General Basrah Hospital by (AluI, DraI, ScaI) restriction enzymes.The genetic restriction map was detected by determining the locations of a number of restriction endonuclease cleavage sites on the DNA relative to each other. AluI enzyme was showed three bands (100 bp, 90 bp and 60 bp) revealing there are two sites of this enzyme, on the other hand DraI, ScaI gave two bands (125 bp) for each enzyme revealing there was a single site of these enzymes, A variety of tcdB gene which detected in this study a new modification increasing the virulence of A^-B^+ isolates of C. difficile with the large defect of tcdA gene.*

I. Introduction:

Clostridium difficile has been recognized as acause of nosocomial diarrhea and pseudomembranous colitis. The enteropathogenicity is associated with production of both toxin A and toxin B [1], but an increasing number of report mention infections due to toxin A- negative toxin B- positive (A^-B^+) strains [5]. Toxin A and toxin B are encoded by *tcdA* and *tcdB* genes respectively, and are located in ~19.6-kb Pathogenicity Locus (PaLoc) in single open reading frames [1]. *tcdA, tcdB, tcdR, tcdC, and tcdE* are PaLoc genes that play key roles for encoding, regulating, and releasing toxin A and toxin B [3].

Two types of toxin A^-/B^+ strains have been identified: strain 8864 and strain 1470 [7]. Both are truncated in the repetitive 3´-end domain, with 8864 having a 6.0 kb deletion [15] and it has been suggested that the production of a variant toxin is associated with its enteropathogenicity [7]. This variant toxin (B) seems more potent than toxin B of 10463 [15], and strain 1470 having a 1.7 kb deletion in 3´-end domain [12], this domain encodes the carboxy repetitive oligopeptide region that contains both the binding site to cell receptor [17]. Toxinotype is mainly determined by analyzing amplified PCR fragments of toxin gene after digesting with restriction enzymes by restriction fragment length polymorphisms (RFLPs) [12]. Currently, there are twenty seven different toxinotypes (I to XXVII) of *C. difficile* and this number keeps growing. These variation types have evolved due to point mutations, deletion or insertion within the Pathogenic Locus [13].

Here we report the use of restriction enzymes to digestive a segment for the nonrepeating sequences of the toxin B gene which amplified by primer NK104-NK105 to determine genetic restriction map for A^-B^+ isolate in *C. difficile*.

II. Materials and Methods:

Bacterial collection and isolation. Watery stool specimens (n=200) were collected from February to July -2010 from General Basrah Hospital. Equal volumes of stool specimen and absolute ethanol were mixed and incubated at room temperature for 30 min. The alcohol-treated stool specimen was inoculated onto anaerobically reduced cycloserine-cefoxitin fructose agar (CCFA), and it was anaerobically incubated at 37ºC in an anaerobic jar for 48 to 72 h [9]. All bacteria used in this study were identified by odor and colony morphology on CCFA and cell morphology after Gram staining.

Isolation of DNA. Genomic DNAs of bacteria were prepared by the method of Kato [6], with modification of buffer type which was replaced TES buffer to TE buffer [16]. Asingle colony was suspended in 50 µl of TE (1.214 gm Tri-HCl, 1.86 gm EDTA) and suspension was heated at 95° C for 10 min and suspension was centrifuged at 15000 xg for 2 min.

PCR assay. PCR assay was performed by *16S rRNA* for identification of *C. difficile* by Penders [10], and two primers sets were used to detect the toxin A gene and toxin B gene; primer NK9 and NK11 was derived from repeating portion of *C. difficile* toxin A gene and primer NK104 and NK105 was derived from nonrepeating sequences of *C. difficile* toxin B gene(table 1) .PCRs carried out in 25 µL containing 5µL of DNA tamplate ,1µL of each forward and reverse primers,12µL master mix (table 2). The thermal profile for primer pair NK9-NK11 was 35 cycles comprising 95º C for 20 sec. and 62º C for 120 sec. while PCR amplification with primer pair NK104-NK105 was performed for 35 cycles, consisting of 95º C for 20 sec. and 55º C for 120 sec. At the conclusion of the PCR cycles, the tubes were incubated at 74º C for 5 min [6].

Table (1) Sequences of the PCR primers

Primers		Sequence	Length nt.	Tm°
16S rRNA	F*	5'-TTGAGCGATTTACTTCGGTAAAGA-3'	24	58
	R*	5'-CCATCCTGTACTGGCTCACCT-3'	21	59
NK9	F	5'-CCACCAGCTGCAGCCATA-3'	18	58
NK11	R	5'-TGATGCTAATAATGAATCTAAAATGGTAAC-3'	30	76
NK104	F	5'-GTGTAGCAATGAAAGTCCAAGTTTACGC-3'	28	80
NK105	26	5'-CACTTAGCTCTTTGATTGCTGCACCT-3'	R	76

F* :Forward. R* :Reverse. Tm^{o}: Melting temperature.

Table (2) Amount of PCR reaction

Chemicals	Volume
Master Mix	12.5 µl
Premier Forward	1 µl
Premier Reverse	1 µl
DNA	5 µl
Sterile deionized water	5.5 µl

Genetic restriction map. The genetic restriction map of NK104-NK105 gene fragment for $A^{-}B^{+}$ isolate was derived by determining the locations of a number of restriction endonuclease cleavage sites on the DNA by (*Alu*I, *Dra*I, *Sca*I) restriction enzymes.

The reaction was prepared with 10µL of DNA tamplate, Restriction Enzyme 2µL (table 3) for restriction digestion by mixing the first four reagents by pipetting them in eppendorf tube, and then added the restriction enzyme. The reaction was incubated at 37° C for 24 h. The Multi-Core buffer which is designed of RE 10X buffer for broad compatibility with many restriction enzymes.

Table (3) Amount of restriction digestion reaction

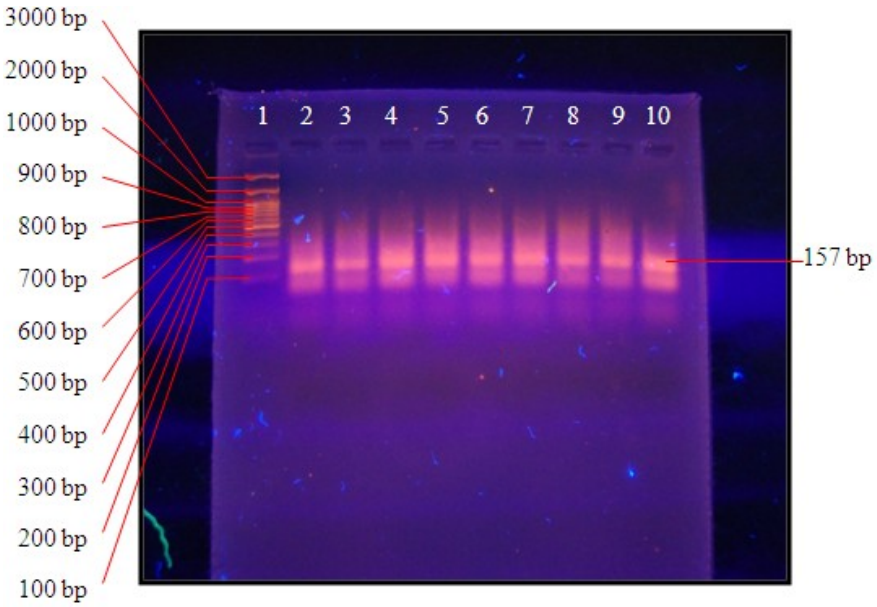
Chemicals	Volume
Sterile, deionized water	7.3 µl
RE 10X Buffer	2 µl
Acetylated BSA, 10 µg/ µl	0.2 µl
DNA template	10 µl
Restriction Enzyme, 10 U/ µl	0.5 µl
Final Volum	20 µl

III. Results and Discussion:

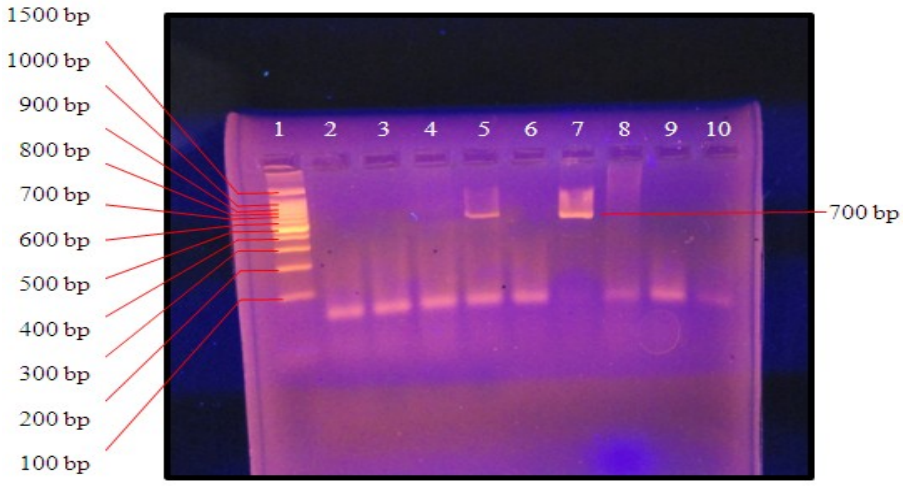
Morphological identification. In this study 200 samples of stool were collected from patients with diarrhea,16 (8%) of these samples were classified as *C. difficile* according to morphological characteristics on CCFA which consist; colonies have a distinct horse stable-like odor [14], large colonies and slightly flat and circular with filamentous edge. The microscopic characteristics observed by Gram stain which consist long, thin, straight rods with oval subterminal spores [2].

Molecular base of *16S rRNA* gene detection by PCR. The results of PCR products of *16S rRNA* gene amplification revealed the presence of a 157 bp product when compared to the molecular ladder, thus identifying the isolates as *C. difficile* as shown in fig (1), and this result agrees with Mutters [8]. It has also been suggested that *16S rRNA* was chosen for the detection of *C. difficile* because it exhibits a high degree of functional and evolutionary homology within all bacteria and these sequences have been used for phylogenetic comparisons and classifications of microbial organisms [4].

Differentiation of the toxigenic group of *C. difficile* with PCR. PCR can be used to differentiate toxigenic group of *C. difficile* with NK9-NK11 primer derived from the repeating portion of the toxin A gene. By this primer we observed a shorter segment of approximately 700 bp for six isolates as shown in figure (2), these isolates can be classified as $A^{-}B^{+}$ group according to Kato [6].

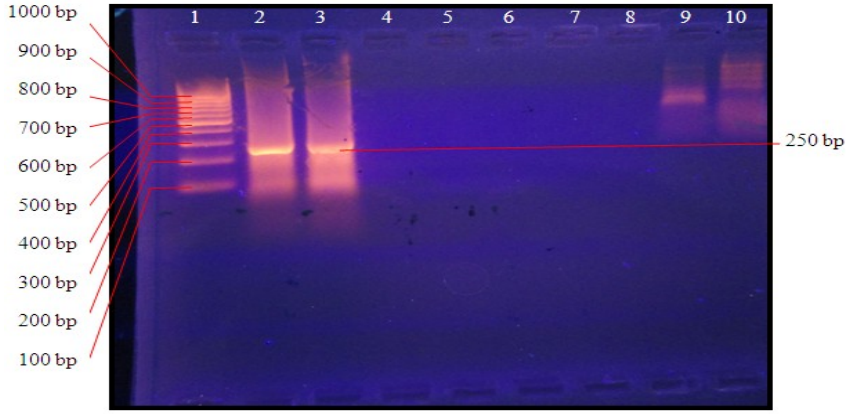


Fig(1): PCR product for *16S rRNA* based primers gave 157 bp band on agarose gel corresponding when compared to the
molecular ladder. Lane:(1) molecular ladder (3000-100) bp, lanes: (2-10) bands of PCR product for *C. difficile* with *16S rRNA*.



Fig(2): PCR product with NK9-NK11 primer which gave700 bp band on agarose gel corresponding when compared to the molecular ladder. Lane:(1) molecular ladder (1500-100) bp, lanes:(5,7) bands of PCR product for *C. difficile* with NK9-NK11.

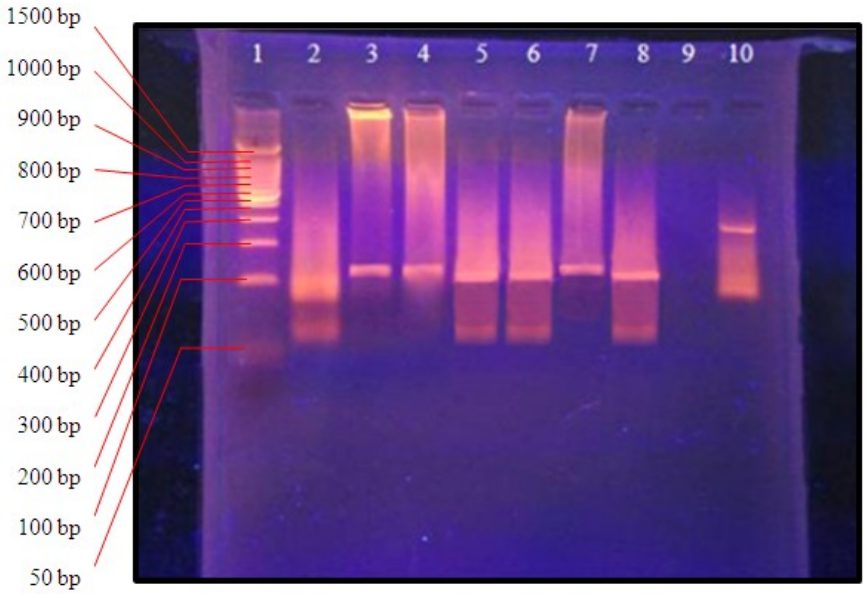
Using PCR with NK104-NK105 primer derived from nonrepeating sequences of the toxin B gene, the isolates which classified as A^-B^+ with NK9-NK11 that gave product approximately 250 bp with primer NK104-NK105 as shown in (fig.3) this product is in agreement with study performed by Kato [6].



Fig(3): PCR product with NK104-NK105 primer which gave 250 bp band on agarose gel corresponding when compared to the molecular ladder. Lane:(1) molecular ladder (1500-100) bp, lanes:(2,3) bands of PCR product for *C. difficile* with NK04-NK105.

From these results we can conclude that the insertion with NK104-NK105 primer for A^-B^+ isolates perhaps compensate the defect in deletion of repetitive region in toxin A gene of A^-B^+ of *C. difficile* because Lyerly [7] suggested that toxin B from A^-B^+ was more lethal than toxin A^+B^+ were comparable in their cytotoxic activities.

Restriction map. In the present study the restriction map for DNA fragment with 250 bp of no. 55 isolate which classified as A^-B^+ was determined by (*Alu*I, *Dra*I, *Sca*I) restriction enzymes. At first this fragment is restricted by *Alu*I which gave three bands sized (60 bp, 90 bp and 100 bp) we must concluded that there are two restriction sites for *Alu*I, whereas two fragments that each fragment sized (125 bp) were produced by each enzyme *Dra*I and *Sca*I we must concluded that there is one restriction site for *Dra*I, *Sca*I. These results disagrees with (GenBank: X53138.1 GI: 40442) which consist of two fragments with *Alu*I sized (7 bp, 197 bp) and two fragments with each of *Dra*I, *Sca*I sized (49 bp,155 bp) and (41 bp, 163 bp) respectively. Restriction with mixture of two enzymes *Alu*I and *Dra*I yield three fragments sized (60 bp, 65 bp and 100 bp) it is difficult to distinguish between two bands sized (60 bp, 65 bp), so nearly similar in size that they have not been separated by electrophoresis [11]. The mixture of *Alu*I and *Sca*I yield three bands sized (60 bp, 65 bp and 100 bp), while the mixture of *Dra*I and *Sca*I generated two fragments (125 bp) whereas the mixture of *Alu*I, *Dra*I, *Sca*I yield three fragments sized (60 bp, 65 bp and 100 bp) as shown in figure (4).

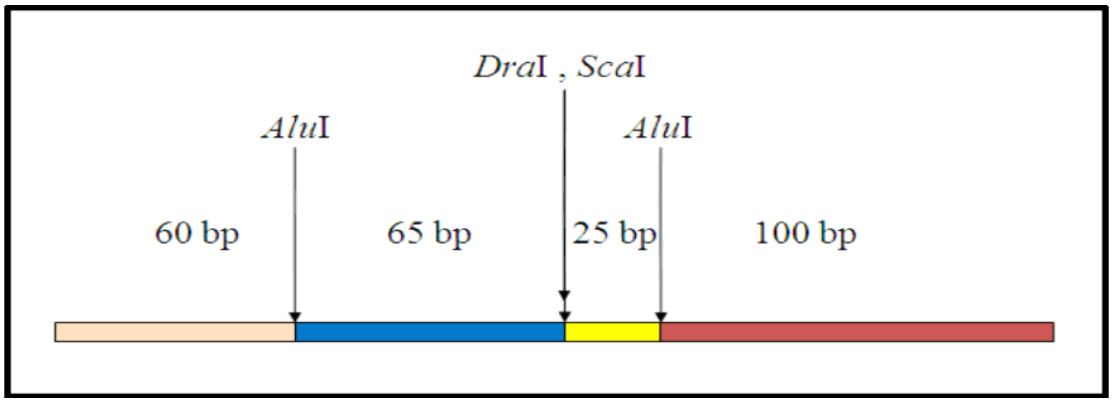


Fig(4): Restriction enzymes of NK104-NK105 gene fragment. Lane:(1) molecular ladder (1500-50) bp, lanes:(2) bands of *Alu*I, lane:(3) bands of *Dra*I, lane:(4) bands of *Sca*I, lane:(5) bands of *Alu*I and *Dra*I, lane:(6) bands of *Alu*I and *Sca*I, lane:(7) bands of *Dra*I and *Sca*I, lane:(8) bands of *Alu*I, *Dra*I and *Sca*I, lane:(10) undigestive DNA fragment with 250 bp.

The restriction map can be determined from these results (fig.5). These results showed that these differences in size and number of DNA fragment which digested with restriction enzymes in comparison with (GenBank: X53138.1 GI: 40442).

In conclusion, the variation in numbers and locations of bands that produced by restriction enzymes of A^-B^+ isolate perhaps reflect the higher modification in toxin B gene of A^-B^+ which responsible of virulent of A^-B^+ strains of *C. difficile* with defect of repeating region for toxin A gene.

Further studies should be performed to detection the real reasons that to lead to prevalence A^-B^+ strains in Basrah and determine the effect of variant toxin B in virulence of *C. difficile*.



Fig(5): Restriction map of DNA fragment with 250 bp for nonrepeating region of *tcd*B gene for A^-B^+ isolate which was amplified by NK104-NK105.

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