



## ***Corynebacterium Diphtheriae*: A Case Report of Some Isolates in National Ear Care Centre Kaduna**

**Maryam Ibrahim Rimi<sup>1\*</sup>, Isaac Agyigraa<sup>1,2</sup>, Kelechi N. Nkwazema<sup>1</sup>,  
Abubakar Yakubu Jajere<sup>1</sup>, Astaharam Ardo Jabbo<sup>1</sup>, Sumayya Ibrahim Ango<sup>1</sup>  
and Muhammad Kabir<sup>1</sup>**

<sup>1</sup>Department of Medical Laboratory Service, National Ear Care Centre, P.M.B. 2438, Kaduna, Nigeria.

<sup>2</sup>Department of Pharmacology and Therapeutics, Ahmadu Bello University Zaria, Kaduna, Nigeria.

### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author MIR designed the study, wrote the protocol and wrote the initial draft of the manuscript. Authors AYJ and SIA performed the statistical analysis. Authors IA and KNN managed the analyses of the study. Authors MIR, AAJ and MK managed the literature searches. All authors read and approved the final manuscript.*

### **Article Information**

DOI: 10.9734/AJRID/2019/v2i430115

Editor(s):

(1) Dr. Shweta Sharma, Department of Microbiology, Dr. Ram Manohar Lohia Hospital and PGIMER, New Delhi, India.

Reviewers:

(1) Constantino Gil Juárez, Benemérita Universidad Autónoma de Puebla, México.

(2) yed Umer Jan, University of Balochistan, Pakistan.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/52699>

**Case Study**

**Received 17 September 2019**

**Accepted 21 November 2019**

**Published 26 November 2019**

### **ABSTRACT**

This is a case study of the incidence of diphtheria in National Ear Care Centre Kaduna. Samples included all patients presented with diphtheria symptoms referred to the hospital from October to December; 2018. A total of 8 patients were referred and several samples taken for laboratory investigations. 8 throat swabs were collected for organism isolation by inoculation on some certain agars, incubation, secondary gram and microscopy; colonies appeared to be biconcave, large and whitish. Microscopy revealed characteristic Chinese lettered, pleomorphic bacillus which are features of *Corynebacterium diphtheriae*. Blood samples were used for urea and creatinine estimation which yielded abnormal high values in all except the creatinine of one of the samples indicating kidney impairment which could be attributed to presence of the tox gene. To further confirm the incidence of the disease; one throat swab was saved at 4°C for molecular confirmation and characterisation of the gene. It was then transported to Molecular laboratory and the DNA

\*Corresponding author: E-mail: [Maryambdw@icloud.com](mailto:Maryambdw@icloud.com);

extracted using the phenol/chloroform extraction method. Both the A and B subunits of the tox gene were amplified from the DNA extract at 248bp and 297bp respectively. Sanger's Dye Terminator Cycle Sequencing (DTCS) of both the subunits of the tox gene was carried out and the sequences were obtained and converted to the fasta format using a Finch TV for bioinformatics analyses using the NCBI blast tool. The A and B subunits showed a percentage identity of 99.59% and 95.57% to *Corynebacterium diphtheriae* mitis strain NCTC 3529 genome assembly, chromosome: 1 respectively. The A subunit also showed 99.59% identity to *Corynebacterium diphtheriae* strain BQ11 chromosome; complete genome while the B subunit also showed 95.57% identity to *Corynebacterium diphtheriae* mitis strain NCTC 7838 genome assembly chromosome: 1. A mutation was also discovered in the tox genes as indicated by the E-value of  $3e-84$  and  $2e-121$  for A and B subunits respectively which are values that are less than 1. This is also confirmed by 5 gaps noticed from the blast results (4 gaps from A and 1 from B) indicating the presence of indels. In conclusion; there are confirmed incidences and sporadic cases of diphtheria in Kaduna State and the organism is said to have mutated.

**Keywords:** DTx/DT: Diphtheria Toxin; Cds: Coding sequence; Tox: Toxigenic/toxin; DTxR: Diphtheria Toxin Repressor; DTP/DPT: Diphtheria; Pertussis; Tetanus; BV: Biovar; Blast: Basic local alignment search tool.

## 1. INTRODUCTION

Diphtheria is a potentially life-threatening upper respiratory infection caused by *Corynebacterium diphtheriae* which has been largely eradicated in developed nations since the introduction of childhood vaccination programs, but is still reported in the Third World and increasingly in some areas in Eastern Europe. Antibiotics are effective in the early stages, but recovery is generally slow [1].

*Corynebacterium diphtheriae*; this is the pathogenic bacterium that causes diphtheria. It is also known as the Klebs-Löffler bacillus, because it was discovered in 1884 by German bacteriologists Edwin Klebs (1834–1912) and Friedrich Löffler (1852–1915) [2]. The genome of *C. diphtheriae* consists of a single circular chromosome of 2,5 Mbp, with no plasmids [3,4]. The genome shows an extreme compositional bias, being noticeably higher in G+C near the origin than at the terminus. The bacterium is sensitive to the majority of antibiotics [5].

DT gene: Diphtheria toxin (DT) is an extracellular protein of *Corynebacterium diphtheriae* that inhibits protein synthesis and kills susceptible cells [6]. The gene that encodes DT (*tox*) is present in some corynephages, and DT is only produced by *C. diphtheriae* isolates that harbor *tox<sup>+</sup>* phages. The diphtheria toxin repressor (DtxR) is a global regulatory protein that uses  $Fe^{2+}$  as co-repressor. Holo-DtxR represses production of DT, corynebacterial siderophore, heme oxygenase, and several other proteins. Diagnostic tests for toxinogenicity of *C.*

*diphtheriae* are based either on immunoassays or on bioassays for DT [7]. Molecular analysis of *tox* and *dtxR* genes in recent clinical isolates of *C. diphtheriae* revealed several *tox* alleles that encode identical DT proteins and multiple *dtxR* alleles that encode five variants of DtxR protein. Therefore, recent clinical isolates of *C. diphtheriae* produce a single antigenic type of DT, and diphtheria toxoid continues to be an effective vaccine for immunization against diphtheria [2].

## 1.2 Purpose of Study

The purpose of the study is to report and prove that there are sporadic cases of diphtheria in Kaduna State and to characterise the DTx gene using molecular techniques, bioinformatics and homology searches.

## 2. MATERIALS AND METHODS

### 2.1 Description of the Study Area

This study was conducted in National Ear Care Centre; Kaduna State, Nigeria. Kaduna State is located at Northwest of Nigeria; it covers a total area of 46,053 km<sup>2</sup> (17,781 sq mi) and an area rank of 4<sup>th</sup> of the 36 states of Nigeria. It has a population of 6,066,562 people going by 2006 census leaving it the 3<sup>rd</sup> of the 36 states of Nigeria in rank and a density of 130 km<sup>2</sup> (340/sq mi). Its coordinates are 10°20'N and 7°45'E; these coordinates clearly indicate that the location is centralised and connects the major routes reaching most of the states of the Nation. Kaduna State, north central Nigeria, is politically

classified as belonging to the now 'North - West' zone of the current six (6) Geo - political zones. It is populated by about 59 to 63 different ethnic groups if not more [8] and NOG, 2007); where Gwari, Hausa and Fulani are the dominant ethnic groups. Its water supply is sourced through damping of rivers and digging of wells and boreholes. Kaduna State consists of twenty-three (23) Local Government Areas.

## 2.2 Sample Population

The population included all patients presented with diphtheria symptoms in the hospital from October to December; 2018.

**Table 1. Urea estimation protocol**

Reagents	Blank	Standard	Test
Urea reagent 1	100 ul	100 ul	100 ul
Standard reagent	---	10ul	---
Serum Sample	---	---	10 ul
Urea reagent 2	2000 ul	2000 ul	2000 ul
Urea reagent 3	2000 ul	2000 ul	2000 ul

**Table 2. Creatinine estimation protocol**

Reagents	Blank	Standard	Test
Creatinine reagent 1	500 ul	500 ul	500 ul
Creatinine reagent 2	500 ul	500 ul	500 ul
Standard reagent	---	100 ul	---
Serum sample	---	---	100 ul

## 2.3 Sample Collection and Transportation

One clinical throat swab was saved after collection from patients with suspected diphtheria in the Centre and was transported to DNA laboratory and Molecular Research Institute in Sterile water at 4°C.

## 2.4 Bacterial Strains and Processing of Clinical Samples

**Control:** water was used as the negative control during PCR and the molecular mark of 248bp and 297 bp band appearance served as the positive control

**Specimen Collection and Processing:** A total of 8 samples for throat swabs were collected from patients with suspected diphtheria from October to December 2018; cotton wool swab sticks were used for the collection from the throat and were then used for isolation of organism in microbiology unit by primary gram, inoculation on blood agar, chocolate agar and McConkey agar

and incubation at 37°C for 24 hours; secondary gram and microscopy. Blood samples were collected for urea and creatinine estimation from harvested serum in Chemical Pathology unit through the following protocol:

Filled tubes were then incubated for 15minutes and absorbance measured at 600wavelength after blanking with reagent blank

### Calculation:

Absorbance of test/Absorbance of standard \*concentration of standard (13.5)

Absorbance was taking for each after exactly 2 minutes using a stop watch at 340 wavelengths.

### Calculation:

Absorbance of test/ Absorbance of Standard \*Concentration of standard (100).

\*Check appendix III for reagent composition.

Some of the patients underwent surgery to remove a membrane on the pharynx from the theatre that was taken to ABUTH; Shika for histological analyses using the following protocol:

- Samples were fixed in formalin and transported to ABUTH; Shika for histological analyses.
- They were then grossed using ruler, scalpel blade, pencil for labelling and inserted into cassettes for automatic tissue processing.
- They were then inserted and arranged in an automatic tissue processor and passed through the following reagents to confer rigidity for microtomy: 70% alcohol, 90% alcohol, Absolute alcohol (2 changes) and xylene (3 changes) for 2 hours each.
- They were then impregnated with liquid paraffin wax and embedded in molten paraffin wax in the embedding chamber.
- They were sectioned and stained using the H and E staining technique.
- They were mounted with DPX and presented to the consultant pathologist for diagnoses.

Finally; one clinical throat swab was packed in sterile water package for transport to DNA lab Kaduna for further investigations according to following explained methodology.

## 2.5 Isolation and Purification of DNA from the Swabs

This was achieved using the phenol/ chloroform extraction method described by Kirby K. S as follows:

- Vials or Eppendorf tubes were first labelled to avoid mix up.
- Exactly 200 ul of sterile water sample (containing cells from the swab) was dispensed in a 1.5 ml tube.
- Exactly 400 ul of lysis buffer and 10 ul of proteinase K was added.
- Tubes were then vortexed and placed on heat block at 65°C for 1 hour for incubation
- A total 400 ul of phenol/ chloroform of ratio 1:1 was added and vortexed; it was then spun at 14000 rpm for 10 minutes.
- Supernatant was then pipetted out and introduced to new 1.5 ml tubes.
- Exactly 400 ul of chloroform was added to the recovered layer and vortexed; it was then spun for 5 minutes and supernatant carefully removed and introduced to new tube.
- A total 1000 ul of absolute ethanol was added and 40 ul of 3 M sodium acetate was added and stored at -20°C in freezer for overnight incubation.
- It was then centrifuged in cold centrifuge at 14,000 rpm for 10 minutes and supernatant decanted.
- Exactly 400 ul of 70% ethanol was added to the debris and re-centrifuged for 5 minutes in the cool/freezing centrifuge at 4°C.
- Supernatant was then decanted for sole purpose of drying out the ethanol; it was re-centrifuged at 14,000 rpm and removed using a micro-pipette.
- The extracted DNA was then dried out at room temperature for 20 minutes and stored at -20°C for PCR.

## 2.6 Primer Reconstitution Stage

Primers were designed as stated in Table 2 but procured by Bioneer Company.

The primers were reconstituted by first of all spinning for 1 minute in a safety cabinet and the following procedure followed:

Subunit A- 109.2 ul of distilled water was added to tox 1 and 106.7 ul of distilled water was added to tox 2 using micropipette and both were then vortexed.

Subunit B- 150.2 ul of distilled water was added to Diphth 6 F and 149 ul of distilled water was added to Diphth6R using micropipette and were then vortexed.

**Note:** the amount of distilled water added was indicated on the primer containers.

90 ul of water was added to 4 Eppendorf tubes to which 5 ul of each tox 1, tox 2, diphth6F and diphth6R was added and vortexed.

Thus the primers were ready for use in the PCR.

## 2.7 Amplification of the Tox gene (DT gene)

The polymerase chain reaction (PCR) amplification of the DT gene was conducted according to the method of Nakao and Popovic [9,10].

### First PCR Reaction

This was performed in a programmable DNA Thermal Cycler PTC-100 with a total of 2 ul of DNA solution and the following master mix of Accupower hot start PCR premix by Bioneer Company:

- 1 ul of reconstituted tox1 and tox2 primers
- 1.5 mM MgCl<sub>2</sub>,
- 250 ul of deoxynucleoside triphosphate mixture (1 mM each of total dNTP), and
- 1 U of *Taq* DNA polymerase
- 1X PCR buffer
- Sterile water was then added to make up to 20 ul.
- A negative control was set alongside the samples; just that in the case of negative control, a DNA template was not added, primers and distilled water were added to make up a volume of 20 ul and the lyophilized blue pellet was dissolved by vortexing for 1 minute.
- The thermal cycler was set for pre denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, extension at 72°C for 5 minutes and final extension at 72°C for 5 minutes and it went on for 35 cycles.

### Second PCR reaction

- The above procedure in the first PCR was applied to the sample DNA but the only difference was that the 2<sup>nd</sup> set of primers

Diphth6F and Diphth6R for amplification of the beta subunit of the tox gene were used and annealing temperature of 48°C.

### Visualisation of Amplicon

- A total of 1.5 g of QDLE Agarose was dissolved in 100 ml of distilled water and heated at 30 seconds interval using the highest heat in the microwave until it was completely dissolved and 8 ul of ethidium bromide was added.
- It was then cooled down to a temperature of 65°C.
- 2 combs were then inserted properly into a cast and the cooled dissolved agarose poured while still molten and allowed to solidify to create wells where the PCR products will be carefully pipetted.
- The cast was then inserted into the electrophoretic tank containing buffer after removal of the combs.
- Exactly 0.8 ul of the PCR products of each sample was mixed with bromophenol blue stain included in the master mix and carefully pipetted into the wells and made to run for 45 minutes for the amplicons to migrate alongside a ladder (DNA marker).
- The amplicon was then finally optimized by repeating some reaction conditions like the annealing temperature to 60°C from 45°C to obtain a clearer band ready for sequencing.

### Dye Terminator Cycle Sequencing With Quick Start Kit

All reagents were kept on ice during the sequencing reactions; the reaction was carried out in a 2.0 ml tube and reagents added according to the following order:

1. Deionised H<sub>2</sub>O 7 ul
2. DNA template 3 ul
3. Primers 2.0 ul
4. DTCS Quick start master mix 8.0 ul

This gave a total of 20 ul. The reaction was mixed and centrifuged briefly at 100 rpm.

The sequencing reaction was set in the PCR machine as below;

Thermal cycling program:

- 96°C for 20 sec to denature the DNA
- 50°C for 20 sec to anneal the DNA strands with both the labelled and normal dNTPs
- 60°C for 4 min to elongate the DNA strands

This was set for 30 cycles followed by holding at 4°C

### Ethanol Precipitation

1. A labelled sterile 0.5 ml tube was prepared for each sample.
2. A fresh stop solution/glycogen mixture was prepared as follows per sequencing reaction: 2 ul of 3 M Sodium acetate, 2 ul of 100 mM Na<sub>2</sub>-EDTA and 1 ul of 20 mg/ml of glycogen (provided in the kit). To each of the labelled tubes; 5 ul of the stop solution/glycogen mixture was added.
3. This sequencing reaction was transferred to each of the appropriately labelled tube and mixed thoroughly.
4. 60ul cold 95% (v/v) ethanol from -20° freezer was added and mixed thoroughly and immediately centrifuged at 14,000 rpm at 4°C for 15 min. the supernatant was carefully removed with a micropipette.(the pellet was visible at these stage).
5. The pellet was rinsed with 200 ul 70% (v/v) ethanol from -20° freezer, centrifuged at 14,000 rpm at 4°C for 2 minutes. The supernatant was then carefully removed with a micropipette.
6. It was then vacuum dried for 10 minutes
7. The sample was re suspended in 40 ul of the sample loading solution (provided in the kit).

**Table 3. Primer design as procured by Bioneer Company**

Gene	Primer sequence (2 sets for both A and B subunits of the gene)	Expected amplicon size	Reference
Diphtheria toxin gene (DT gene)	Set 1: Tox 1 (PRN FORWARD) (ATCCACTTTTAGTGCGAGAACCTTCGTCA) Tox2 (PRN REVERSE)(GAAAACCTTTCTTCGTACCA CGGGACTAA) Set 2: Dipht 6F (PRN FORWARD) (ATACTTCCTGG TATCGGTAGC) and Dipht 6R (PRN REVERSE) (CGAATCTTCAACAGTGTCCA)	248bp for the A subunit and 297bp for the B subunit	Nakao and Popovic; 1997

### Sample Preparation for Loading in to the Instrument

1. The re-suspended samples were transferred to the appropriate wells of the sample plate (PN 609801).
2. Each of the re suspended samples was overlaid with one drop of mineral oil from the kit.
3. The sample plate was loaded into the instrument and the desired method was started.
4. Samples were prepared by dissolving 10ul of sample loading solution.
5. 10 ul of SLS was added to any blank wells in arrow.
6. 250 ul of separation buffer to buffer plate was added.
7. The CEQ machine was then started.
8. "CEQ System" was then clicked on desktop.
9. Direct Control was then clicked on run and then plate accessed: the sample and buffer plates were then put and wetting tray filled.
10. Gel Catridge was released and new on placed on replenish.
11. Samples were then named and blank written in blank wells.
12. Running method was set for LFR-C-500bp. (Seq.test-General).
13. Wells were then highlighted with the samples and analysis method clicked and file saved.
14. Run was pressed and reviewed.
15. Finally "OK" was clicked to begin the sequencing.

## 3. RESULTS

### 3.1 Microbiology Unit Results

Colonies appeared after the incubation which were large; biconcave and whitish on all agars. Microscopy revealed pleomorphic bacilli resembling a characteristic Chinese letter-appearance thus *Corynebacterium diphtheriae* isolated for all samples of the 8 patients presented with pharyngitis.

### 3.2 Chemical Pathology Unit Results

The estimated values of urea and creatinine were very high of all patients except one creatinine value that fell within normal range. The urea values include 20.3, 15.5, 28.9, 11.4, 10.3,9.0,31.8 and 20.1 while the creatinine

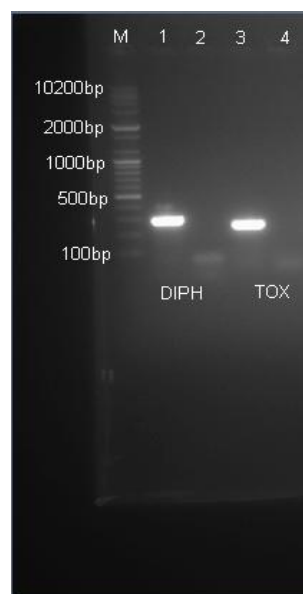
values include 75, 163, 377, 131, 204, 133, 623 and 193.

### 3.3 Histopathology Results

The histopathology report of Oropharyngeal masses showed a grey, brown flat tissue measuring 3x1x0.5cm and solid in nature for the macroscopy while microscopy showed tissue fragments composed of necrotic debris admixed with autolysed tissue exhibiting haemorrhage from ABUTH; Shika.

### 3.4 PCR Results

The PCR results presented in Plate 1 Shows 2 positive PCR results of the DTx.



**Plate 1. M- 1kbp molecular weight marker, lane 1-positive PCR amplicon at 297bp for the B subunit of the DTgene, lanes 2 and 4-negative controls, lane 3- positive PCR amplicon at 248bp for A subunit of DTx gene**  
*\*the PCR result is that of the 52year old patient*

### 3.5 Sequencing Results

The sequence was generally good at about base 18 and was represented by tall distinct peaks that had little overlap; the sequence was of good and high quality accompanied by little stretches of AAAAs as illustrated in the image in appendix I. The sequence belonged to only one product and as such qualified to proceed for bioinformatics analyses (Appendix I).

### 3.6 Sequence for A Subunit of DTx gene

After the sequencing reaction; viewing and editing; the following sequence was obtained for the A-subunit in the fasta format:

```
TTCCTTTTTTAAAAAGGGTATACAAAAGCCAA
ATCTGGTACACAAGGAAATTATGACGATGATT
GGAAAGGGTTTTATAGTACCGACATAAATAC
GACGCTGCGGGATACTCTGGAGATGATGAAA
CCCGCTCTCTGGAAAAGCTGGAGGCGTGGT
CAAAGTGACGTATCCTGGACTGACGAAGTT
CTCGCACTAACAGTGGATAACGC.
```

### 3.7 Sequence for the B Subunit of the DTx Gene

The string of letters below represents sequence for the B-subunit in the fasta format:

```
TGCGTTTTATGCCCGGAGAATACGCGGGAC
GATTATACGAATTATGAACTACTTGAATAAAA
TTGATAACTACTCTACAAAATTATATGCAGC
GAAACCAATATCAACTAGCTCTCCTACCAATG
GAATAGCTTGAGCAACCATTAAAGACGATAA
AGCTATTGATTGTGCCACTATCTCTTCTGTAT
TGTGGTGAACGGCACCGTCTGCAATGCCCAT
TACGCTACCGATACCAGGAATATA.
```

### 3.8 Basic Local Alignment Search Tool (Blast) Results

#### Blast results of A subunit

The A subunit of the gene isolated is a nucleotide with Query ID Icl/Query\_61465 and RID 85E16CKE014. It had a distribution of 99 blast hits on 95 subject sequences from the query sequence and it showed 95.57% identity to both *Corynebacterium diphtheriae* strain NCTC 3529 genome assembly chromosome: 1 (accession number; LR134538.1) and *Corynebacterium diphtheriae* strain NCTC 7838 genome assembly (accession number; LR134537.1). It had a maximum and total score of 322 after covering 95% of the query sequence. The E-value is 3e-84. There were 4 gaps out of the 174bp in the alignment to both strains signifying "indels" which are a type of mutation. The result is summarised in Table 3.

#### Blast results for the B subunit

The blast result for the B subunit is presented in Table 3. The B subunit is more or less similar to the A subunit in its homology searches. It has a query ID of Icl/Query\_162819 and RID

85DC45V4014 with a distribution of 77 blast hits on the subject sequences from the query sequence. It also showed 99.59% identity to both *Corynebacterium diphtheriae* strain NCTC 3529 genome assembly chromosome: 1 (accession number; LR134538.1) and *Corynebacterium diphtheriae* strain BQ11 chromosome complete genome (accession number CP029644.1). It has a total and maximum score of 446 and E-value of 2e-121 out of the 241bp of the B subunit query sequence; 1 gap was noticed signifying indel which is a type of mutation.

## 4. DISCUSSION OF RESULTS

High urea and creatinine values indicate kidney impairment; the causative organism of diphtheria release a potent endotoxin that affects the liver, kidney and other organs. As a results diphtheria patients usually have their urea and creatinine values escalated as the kidneys cannot excrete them due to the action of DTx on them [11]. Out of the 8 throat swabs collected; only one was taken for molecular analysis as a representative of others as they share similar symptoms. It was positive for both the A and B subunits of diphtheria tox (DTx) gene. In the United Kingdom, 3 toxigenic strains of *Corynebacterium diphtheriae* were identified and PCR results showed both A and B subunits of the tox gene in the year 2015 [12]. In a similar study conducted in Atlanta, Georgia; 34 of 36 specimens were positive for one or both subunits of the tox gene using real time PCR assay [13]. In a different report; 170 strains were positive for the A subunit of the DTx gene out of 250 throat swabs obtained from clinical cases and carriers in Moscow, Russia by PCR directed at A subunit of the gene [14]. Similarly; PCR detection of tox gene was positive for the 248bp of the fragment A and the complete tox gene from a specimen obtained from a 38 year old male in a city located in western Canadian province [15,16]. In agreement with the above findings; a study was conducted in Kimba village of Borno State, a cluster of deaths in children following an illness characterized by a swollen neck was reported at Biu General Hospital in Borno State, Nigeria. The village has a population of 1553 people, about 50 km south of the city of Biu. PCR results of pharyngeal swabs were positive for the tox genes confirming the clinical syndrome of diphtheria [17].

The positive sample from the patient was also treated using antibiotics but the symptoms persisted even after administration and infusion with fluids. This is strongly correlated to the

**Table 4. Chemical Pathology parameters of Urea and Creatinine**

Serial number	Hospital number or Lab number	Age	Urea (mmol/L) NR: 2.5-6.5	Creatinine (mmol/L) NR: 9-126
1	112578	9	20.3	75
2	112648	4	15.5	163
3	---	10	28.9	377
4	126075	9	11.4	131
5	126213	52	10.3	204
6	1341	40	9.0	133
7	1225	---	31.8	623
8	113242	30	20.1	193

Key: NR- Normal Range

**Table 5. Summary of Blast Results**

Dtx Gene	Bits	E-Value	1 <sup>st</sup> Organism	2 <sup>nd</sup> Organism	% Homology
A subunit	322	2e-121	<i>Corynebacterium diphtheriae</i> strain NCTC 3529 genome assembly chromosome: 1 (accession number; LR134538.1)	<i>Corynebacterium diphtheriae</i> strain NCTC 7838 genome assembly (accession number; LR134537.1)	95.57 to both organisms
B subunit	446	3e-84	<i>Corynebacterium diphtheriae</i> strain NCTC3529 genome assembly chromosome: 1 (accession number; LR134538.1)	<i>Corynebacterium diphtheriae</i> strain BQ11 chromosome complete genome (accession number CP029644.1)	99.59 to both organisms

\*Check appendix II for photographic presentation of blast results

action of the exotoxin in their system and the unavailability of the antitoxin in the hospital leading to the incomplete treatment of the disease. A similar case was reported in Benin City; Edo state; Nigeria where 9 cases were seen and 3 deaths recorded due to unavailable antitoxin to counteract the effect of the DTx which lead to persistence of symptoms and death [18].

In this study; BLAST results of the A and B subunits that showed the tox gene identifies to *Corynebacterium diphtheriae* Mitis strain NCTC 3529 genome assembly; chromosome: 1 to a degree of 99.59% and 95.57% respectively. The A subunit also showed 99.59% identity to *Corynebacterium diphtheriae* strain BQ11 chromosome; complete genome while the B subunit also showed 95.57% identity to *Corynebacterium diphtheriae* Mitis strain NCTC 7838 genome assembly; chromosome: 1. A similar observation was also made in Borno state; during the outbreak; a sample was taken and PCR, sequencing and bioinformatics analyses was carried out and the tox gene and DTxR of the sample showed *Corynebacterium diphtheriae* biovar *mitis* [17]. In the United Kingdom; genotypically indistinguishable isolates of *C. diphtheriae* var. *Mitis* was also isolated from skin lesions of the index finger of the patient and

a throat swab from an asymptomatic household contact [19]. Similarly; in Canada; a toxigenic *C. diphtheriae* biovar *mitis* strain was recovered from a toe infection of a male patient that travelled to India [20]. Within the NIS; biotype var. *Mitis* isolates were detected during different stages of the epidermis using molecular analyses in the years 1984 through 1995 [21]. Lastly; *C. diphtheriae* var. *Mitis* was also isolated in a cutaneous infection in Indonesia [22].

A total of 5 gaps were noted which signify mutation; 4gaps in the A subunit and 1 in the B subunit. A similar study carried out in Colorado; USA agreed with this finding which presented one silent point mutation in the region of *tox* encoding the A domain of DT, and three silent mutations were detected in the region of *tox* encoding the B domain of DT, but the amino acid sequences of all the DT proteins encoded by these *tox* alleles were identical [23].

The A subunit is more significant than the B subunit because of its higher E value and nearness to zero. Because the E value of 1 assigned to a hit can be interpreted as meaning that in a database of the current size one might expect to see one match with a similar score simply by chance; and the E-value of the A and B subunits are 3e-84 and 2e-121 respectively



which are less than 1; it can be deduced that the tox gene isolated has mutated. This is also confirmed by the total sum of 5 gaps noticed from the blast results (4 gaps from the A and 1 gap from the B subunit) indicating the presence of “indels”.

## 5. CONCLUSION AND RECOMMENDATION

It can therefore be concluded that there are sporadic cases of diphtheria in Kaduna State and molecular techniques reveal that the DTx gene isolated is homologous to the *C. diphtheriae* NCTC strain 3529, *C. diphtheriae* strain NCTC 7838 and *C. diphtheriae* strain BQ11. Finally; molecular analyses and consequent homology searches show that the organism has mutated because of the E-value and presence of indels. It is highly recommended that Diphtheria antitoxin be purchased in all hospitals to complete treatment, vaccines and their boosters should be taken serious. More researches should be carried out to know the point of mutation and possible production of indigenous vaccines.

## CONSENT

As per international standard, patient’s written consent has been collected and preserved by the author(s).

## ETHICAL APPROVAL

The request for the ethical clearance was made to the The Medical Director through. The Acting Head of Clinical Services; Research and Training; National Ear Care Centre; Kaduna. Since the research was timely, worthy and has direct relation with some of the problems in Kaduna and Nigeria as whole, it was approved.

## ACKNOWLEDGEMENT

Authors appreciate the assistance of all the laboratory staff and the management of National Ear Care Centre as well as DNA labs Kaduna.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## REFERENCES

1. Aravena-Roman M, Bowman R, Neill G. Polymerase chain reaction for the

detection of toxigenic *Corynebacterium diphtheriae*. Textbook of Pathology. 1995; 27:71-3.

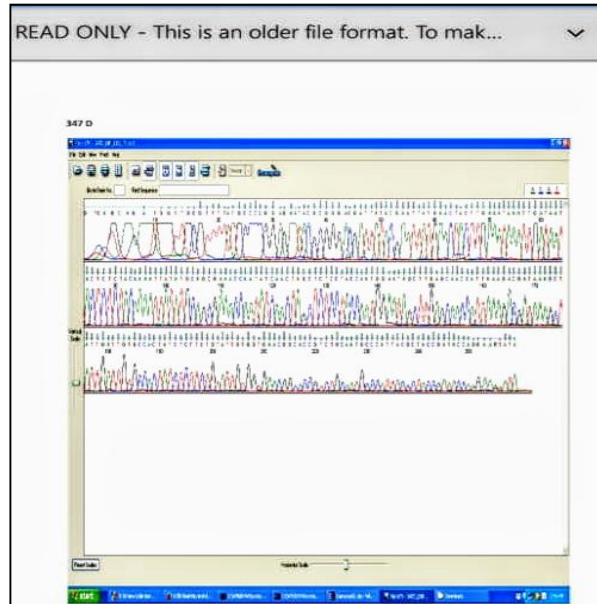
2. Klebs E. III. Sitzung: Ueber Diphtherie (Third session: On diphtheria), Verhandlungen des Congresses für innere Medicin. Zweiter Congress gehalten zu Wiesbaden, 18.-23. (Proceedings of the congress on internal medicine. Second congress held at Wiesbaden. 1883;2:139-154.
3. Cerdeño-Tárraga AM, Efstratiou A, Dover LG, Holden MT, Pallen M, Bentley SD, Besra GS, Churcher C, James KD, De Zoysa A, Chillingworth T, Cronin A, Dowd L, Feltwell T, Hamlin N, Holroyd S, Jagels K, Moule S, Quail MA, Rabinowitsch E, Rutherford KM, Thomson NR, Unwin L, Whitehead S, Barrell BG, Parkhill J. The complete genome sequence and analysis of *Corynebacterium diphtheriae*. Nucleic Acids Research. 2003;22:6516–23.
4. Sangal V, Tucker NP, Burkovski A, Hoskisson PA. The draft genome sequence of *Corynebacterium diphtheriae* bv. Mitis NCTC 3529 reveals significant diversity between the primary disease-causing biovars. Journal of Bacteriology. 2012;194(12):3269. PMID 22628502.
5. Chakraborty SM, Choudghuri AB. Isolation of toxigenic strains of *C. diphtheriae* from skin lesions of children in a rural West Bengal community. Indian Journal of Medical Research. 1969;57:1651-8.
6. Atkinson W, Hamborsky J, McIntyre L, Wolfe S. Diphtheria. Epidemiology And Prevention of Vaccine-Preventable Diseases (10 ed.). Public Health Foundation. 2007;59–70.
7. Behrman RE, Kliegman RM, Jenson HB, Sanders WB. *Corynebacterium diphtheriae* Nelson textbook of Paediatrics. 2000;838-842.
8. Fletcher B, Dan C. Africa. Sir Banister Fletcher’s History of Architecture. Architectural Press. 1996;1466. ISBN 0-7506-2267-9 Available:<http://www.who.int/mediacentre/factsheets/fs089/en/>
9. Nakao H, Popovic T. Development of a direct PCR for detection of the diphtheria toxin gene. Journal of Clinical Microbiology. 1997;7:1651–1655.
10. Nakao H, Popovic T. Development of a direct PCR for detection of the diphtheria

- toxin gene. Journal of Clinical Microbiology. 1997;35:1651-5.
11. Youwang Y, Jianming D, Yong X, Pong Z. Epidemiological features of an outbreak of diphtheria and its control with diphtheria toxoid immunization. International journal of Epidemiology. 1992;21(4).
  12. Public Health England. Infection report/immunization. Diphtheria in England and Wales; Health Protection Weekly Report. 2016;10(13).
  13. Elizabeth A, Mothershed R, Pamela K, Cassidy B, Kevin P, Leonard W, Mayer L, Tanja P. Meningitis and special pathogens branch, division of bacterial and mycotic diseases, national center for infectious diseases, centers for disease control and prevention. Atlanta, Georgia. 2000;30333.
  14. Mikhailovich VM, Melnikov MG, Mazurova IK. Application of PCR for detection of toxigenic *Corynebacterium diphtheriae* strains isolated during the Russian diphtheria epidemic, 1990 through 1994. Journal of Clinical Microbiology. 1994; 33:3061-3.
  15. Pallen MJ, Hay AJ, Puckey LH, Efstratiou A. Polymerase chain reaction for screening clinical isolates of corynebacteria for the production of diphtheria toxin. Journal of Clinical Pathology. 1994;47:353-6.
  16. Efstratiou A, Engler KH, De Zoysa A. Diagnosis and epidemiology of diphtheria. Methods in molecular medicine. Molecular bacteriology, protocols and clinical applications. Humana Press. 1998;15:191-212.
  17. Besa NC, Coldiron ME, Bakri A, Raji A, Nsuami MJ, Rousseau C, Hurtado N, Porten K. Article of Diphtheria outbreak with high mortality in northeastern Nigeria; 2011.
  18. Sadoh AE, Sadoh WE. Diphtheria mortality in Nigeria: The need to stock diphtheria antitoxin. African Journal of Clinical and Experimental Microbiology. 2011;12(2):82-85. ISBN 1595-689
  19. Public Health Laboratory Service. Diphtheria acquired during a cruise in the Baltic Sea. Communicable Disease Representative CDR Wkly. 1997;7:207.
  20. Snyder JW. Media for detection of *Corynebacterium diphtheria*. 3.11.7. In Garcia L. S. (ed.), Clinical microbiological procedures handbook, 3<sup>rd</sup> Ed. ASM Press, Washington DC. 2016;1.
  21. World Health Organization. Diphtheria; 2016. Available:<http://www.who.int/mediacentre/factsheets/fs089/en/>
  22. Engler KH, Glushkevich T, Mazurova IK, George RC, Efstratiou A. A modified Elek test for detection of toxigenic corynebacteria in the diagnostic laboratory. Journal of Clinical Microbiology. 1997;35: 495–498.
  23. Randall KH. Biology and molecular epidemiology of diphtheria toxin and the *tox* gene. Department of Microbiology, University of Colorado Health Sciences Centre, Denver, Colorado. JID 181: S163; 2000.

## APPENDIX

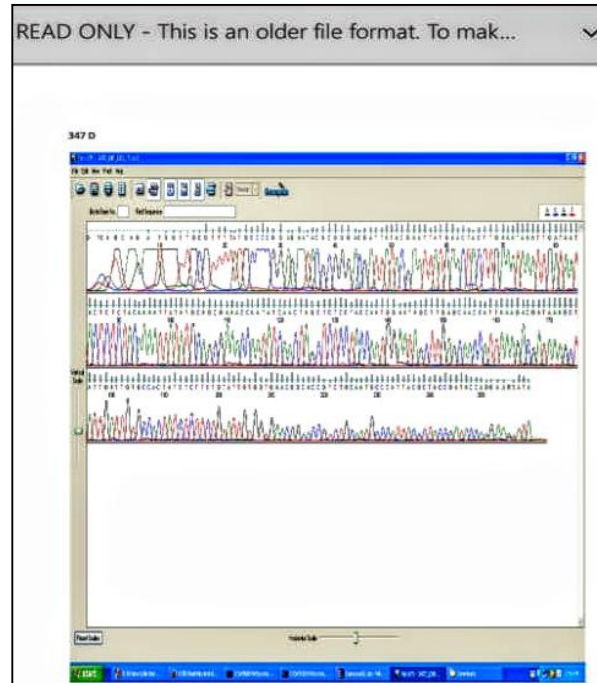
### Appendix I: FINCH TV

- 1) Finch TV A



Finch TV for the A subunit of Diphtheria tox gene

- 2) Finch TV B

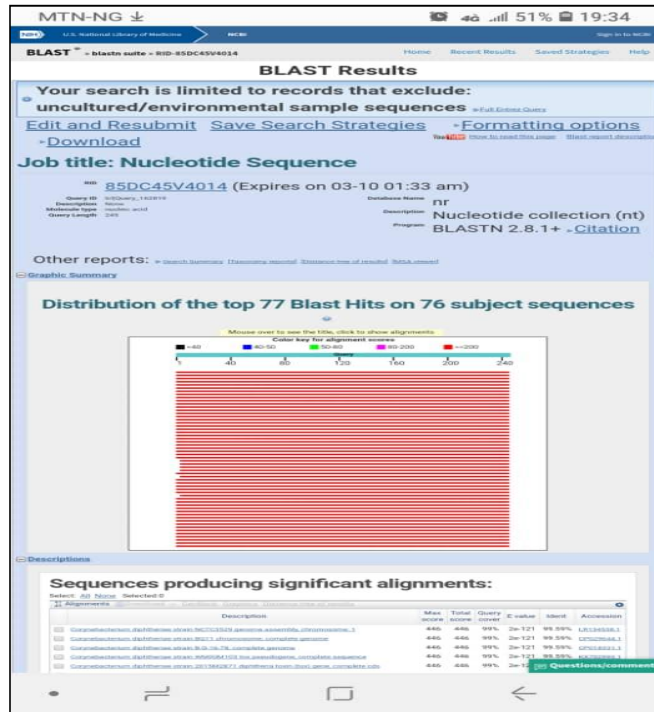


Finch TV for the B subunit of the Diphtheria tox gene

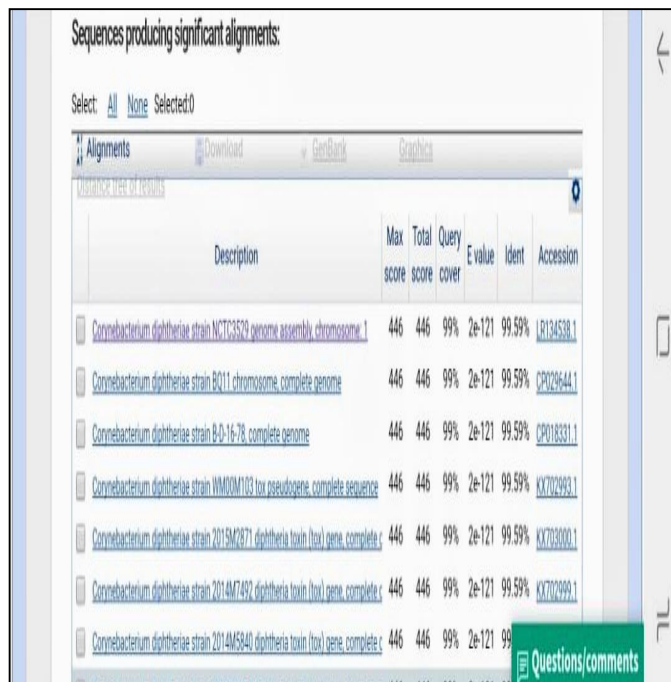
**Appendix II: Photographic presentation of Blast results**

**A) Photographic presentation of nucleotide Blast results of A subunit**

1) Blast results of A subunit of the tox gene and its identification



2) Sequences displaying significant alignment



3) Homology searches of the A subunit of the tox gene

**Corynebacterium diphtheriae strain NCTC3529**  
**genome assembly, chromosome: 1**  
**Sequence ID: [LR134538.1](#) Length: 2528716 Number of Matches:**  
**1**

Range 1: 1942792 to 1944037 [Details](#) [Statistics](#) [Download](#) [Previous](#) [Next](#)

Score	Expect	Identical	Gaps	Strand
446.816(241)	2e-121	244/245(99%)	1/245(0%)	Plus/Minus
Query 1	TGCCTTTTATGCCCGGAGAAATACCGGGACGATTATACGAATTATGAACTACTTGAAT	80		
Sbjct 1944037	TGCCTTTTATGCCCGGAGAAATACCGGGACGATTATACGAATTATGAACTACTTGAAT	1943978		
Query 61	AAATTGATAAATCTCTACAAAATTTATATGCAGCGAAACCAATATCAACTAGCTCTCCT	120		
Sbjct 1943977	AAATTGATAAATCTCTACAAAATTTATATGCAGCGAAACCAATATCAACTAGCTCTCCT	1943918		
Query 121	ACCAATGGAAATAGCTTGAACAACCTTAAAGACGATAAAGCTATTGATTGGCCACTATC	180		
Sbjct 1943917	ACCAATGGAAATAGCTTGAACAACCTTAAAGACGATAAAGCTATTGATTGGCCACTATC	1943858		
Query 181	TCTTCTGATTTGTGTGAACGGCACCTCTGCAATGCCCATTTACCTACCGATACCAGGA	240		
Sbjct 1943857	TCTTCTGATTTGTGTGAACGGCACCTCTGCAATGCCCATTTACCTACCGATACCAGGA	1943798		
Query 241	A-TAT 244			
Sbjct 1943797	AGTAT 1943793			

[Download](#) - [GenBank](#) [Statistics](#) [Next](#) [Previous](#) [Descriptions](#)

**Corynebacterium diphtheriae strain BQ11**  
**chromosome, complete genome**  
**Sequence ID: [CP029644.1](#) Length: 2557381 Number of Matches:**  
**1**

Range 1: 172587 to 172827 [Details](#) [Statistics](#) [Download](#) [Previous](#) [Next](#)

Score	Expect	Identical	Gaps	Strand
446.816(241)	2e-121	244/245(99%)	1/245(0%)	Plus/Minus
Query 1	TGCCTTTTATGCCCGGAGAAATACCGGGACGATTATACGAATTATGAACTACTTGAAT	80		
Sbjct 172827	TGCCTTTTATGCCCGGAGAAATACCGGGACGATTATACGAATTATGAACTACTTGAAT	172768		
Query 61	AAATTGATAAATCTCTACAAAATTTATATGCAGCGAAACCAATATCAACTAGCTCTCCT	120		
Sbjct 172767	AAATTGATAAATCTCTACAAAATTTATATGCAGCGAAACCAATATCAACTAGCTCTCCT	172708		
Query 121	ACCAATGGAAATAGCTTGAACAACCTTAAAGACGATAAAGCTATTGATTGGCCACTATC	180		
Sbjct 172767	ACCAATGGAAATAGCTTGAACAACCTTAAAGACGATAAAGCTATTGATTGGCCACTATC	172648		
Query 181	TCTTCTGATTTGTGTGAACGGCACCTCTGCAATGCCCATTTACCTACCGATACCAGGA	240		
Sbjct 172647	TCTTCTGATTTGTGTGAACGGCACCTCTGCAATGCCCATTTACCTACCGATACCAGGA	172588		
Query 241	A-TAT 244			
Sbjct 172587	AGTAT 172583			

[Download](#) - [GenBank](#) [Statistics](#) [Next](#) [Previous](#) [Descriptions](#)

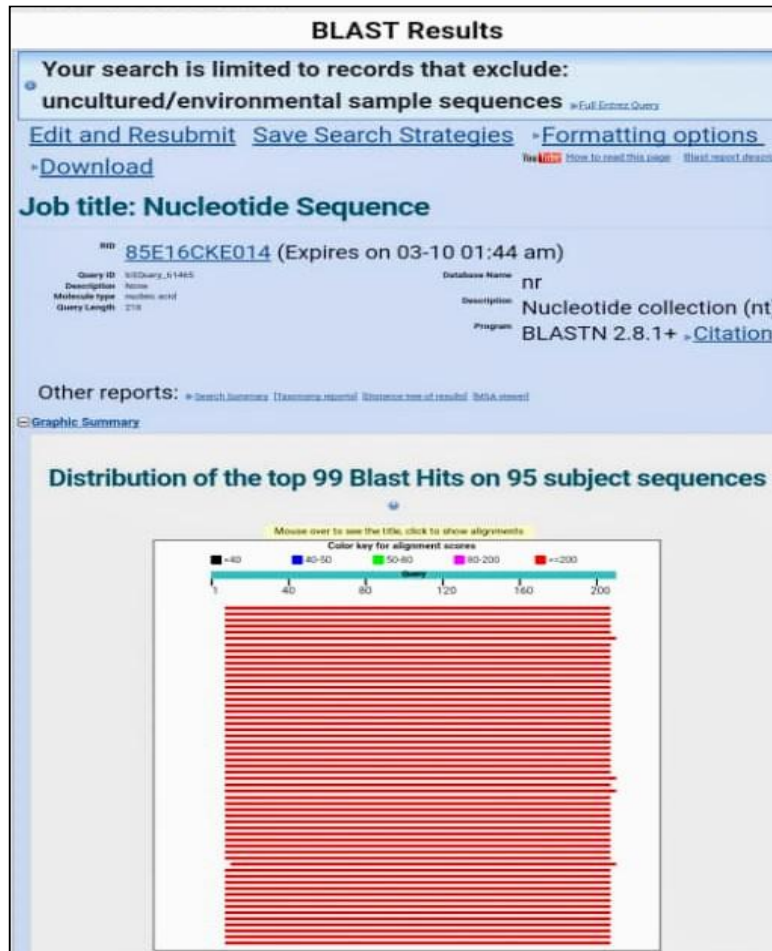
**Corynebacterium diphtheriae strain B-D-16-78,**  
**complete genome**  
**Sequence ID: [CP018331.1](#) Length: 2474151 Number of Matches:**  
**1**

Range 1: 1873704 to 1873994 [Details](#) [Statistics](#) [Download](#) [Previous](#) [Next](#)

Score	Expect	Identical	Gaps	Strand
446.816(241)	2e-121	244/245(99%)	1/245(0%)	Plus/Minus
Query 1	TGCCTTTTATGCCCGGAGAAATACCGGGACGATTATACGAATTATGAACTACTTGAAT	80		
Sbjct 1873950	TGCCTTTTATGCCCGGAGAAATACCGGGACGATTATACGAATTATGAACTACTTGAAT	1873891		
Query 61	AAATTGATAAATCTCTACAAAATTTATATGCAGCGAAACCAATATCAACTAGCTCTCCT	120		
Sbjct 1873890	AAATTGATAAATCTCTACAAAATTTATATGCAGCGAAACCAATATCAACTAGCTCTCCT	1873831		
Query 121	ACCAATGGAAATAGCTTGAACAACCTTAAAGACGATAAAGCTATTGATTGGCCACTATC	180		
Sbjct 1873830	ACCAATGGAAATAGCTTGAACAACCTTAAAGACGATAAAGCTATTGATTGGCCACTATC	1873771		
Query 181	TCTTCTGATTTGTGTGAACGGCACCTCTGCAATGCCCATTTACCTACCGATACCAGGA	240		
Sbjct 1873770	TCTTCTGATTTGTGTGAACGGCACCTCTGCAATGCCCATTTACCTACCGATACCAGGA	1873711		
Query 241	A-TAT 244			
Sbjct 1873710	AGTAT 1873706			

B) Photographic presentation of nucleotide Blast results of B subunit

- 1) Blast results of the B subunit of the tox gene and its identification



3) Sequences displaying significant alignment

**Sequences producing significant alignments:**

Select All None Selected 0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
<a href="#">Cornebacterium diphtheriae strain NC033529 genome assembly chromosome.1</a>	322	322	95%	3e-84	95.57%	LR126538.1
<a href="#">Cornebacterium diphtheriae strain NC033530 genome assembly chromosome.1</a>	322	322	95%	3e-84	95.57%	LR126537.1
<a href="#">Cloning vector pG136784 complete sequence</a>	322	322	95%	3e-84	95.57%	MM199427.1
<a href="#">Cornebacterium diphtheriae strain B011 chromosome complete genome</a>	322	322	95%	3e-84	95.57%	CP010644.1
<a href="#">Cornebacterium diphtheriae strain FGAARG02_187 chromosome complete genome</a>	322	322	95%	3e-84	95.57%	CP0102418.1
<a href="#">Transformation vector pBICAT011 complete sequence</a>	322	644	96%	3e-84	95.57%	KY784937.1
<a href="#">Vector pGSA28-DV3 complete sequence</a>	322	322	95%	3e-84	95.57%	U729421.1
<a href="#">Vector pGSA28-DV1 complete sequence</a>	322	322	95%	3e-84	95.57%	U729420.1
<a href="#">Cornebacterium diphtheriae strain B-2-16-78 complete genome</a>	322	322	95%	3e-84	95.57%	CP010331.1
<a href="#">Cornebacterium diphtheriae strain J9620103 bioassay genome complete sequence</a>	322	322	95%	3e-84	95.57%	KX022923.1
<a href="#">Cornebacterium diphtheriae strain J01562871 diphtheria toxin biosyn. complete cds</a>	322	322	95%	3e-84	95.57%	KX023001.1
<a href="#">Cornebacterium diphtheriae strain J01447491 diphtheria toxin biosyn. complete cds</a>	322	322	95%	3e-84	95.57%	KX023098.1
<a href="#">Cornebacterium diphtheriae strain J01462840 diphtheria toxin biosyn. complete cds</a>	322	322	95%	3e-84	95.57%	KX023098.1
<a href="#">Cornebacterium diphtheriae strain J01342161 diphtheria toxin biosyn. complete cds</a>	322	322	95%	3e-84	95.57%	KX023097.1
<a href="#">Cornebacterium diphtheriae strain J01342777 diphtheria toxin biosyn. complete cds</a>	322	322	95%	3e-84	95.57%	KX023098.1

4) Homology searches of the B subunit of the tox gene

**Corynebacterium diphtheriae strain NCTC3529**  
 genome assembly, chromosome: 1  
 Sequence ID: [LR134538.1](#) Length: 2528716 Number of Matches: 1

Range 1: 1942969 to 1943170 [Search](#) [Display](#)

Score	Expect	Identities	Gaps	Strand
322.981(174)	3e-84	194/203(95%)	4/203(1%)	Plus/Plus

Query 8 TTTAAAAGGGTATACAAAAGCC-AAATCTGGTACACAAAGGAATTTAGCGATGTTGG 86  
 Sbjct 1942969 TTTAAAAGGGTATACAAAAGCC-AAATCTGGTACACAAAGGAATTTAGCGATGTTGG 1943170

Query 67 AAAGGGTTTTATAGTACCCGAC-ATAAATACGACGCTGGGATACCTGGAGATGATG-A 124  
 Sbjct 1943028 AAAGGGTTTTATAGTACCCGAC-ATAAATACGACGCTGGGATACCTGGAGATGATG-A 1943107

Query 125 AACCCCTCTCTGGAAAAGCTGGAAAGCTGGTCAAGTGAAGTATCTGGACTGACGAA 184  
 Sbjct 1943088 AACCCCTCTCTGGAAAAGCTGGAAAGCTGGTCAAGTGAAGTATCTGGACTGACGAA 1943147

Query 185 GTTCTGCACCTAACAGTGGATAA 207  
 Sbjct 1943148 GTTCTGCACCTAACAGTGGATAA 1943170

[Download](#) - [GenBank](#) [Graphics](#) [Next](#) [Previous](#) [Descriptions](#)

**Corynebacterium diphtheriae strain NCTC7838**  
 genome assembly, chromosome: 1  
 Sequence ID: [LR134537.1](#) Length: 2435114 Number of Matches: 1

Range 1: 20514 to 20519 [Search](#) [Display](#)

Score	Expect	Identities	Gaps	Strand
322.981(174)	3e-84	194/203(95%)	4/203(1%)	Plus/Plus

Query 8 TTTAAAAGGGTATACAAAAGCC-AAATCTGGTACACAAAGGAATTTAGCGATGTTGG 86  
 Sbjct 20514 TTTAAAAGGGTATACAAAAGCC-AAATCTGGTACACAAAGGAATTTAGCGATGTTGG 20519

Query 67 AAAGGGTTTTATAGTACCCGAC-ATAAATACGACGCTGGGATACCTGGAGATGATG-A 124  
 Sbjct 20573 AAAGGGTTTTATAGTACCCGAC-ATAAATACGACGCTGGGATACCTGGAGATGATG-A 20582

Query 125 AACCCCTCTCTGGAAAAGCTGGAAAGCTGGTCAAGTGAAGTATCTGGACTGACGAA 184  
 Sbjct 20138 AACCCCTCTCTGGAAAAGCTGGAAAGCTGGTCAAGTGAAGTATCTGGACTGACGAA 20193

Query 185 GTTCTGCACCTAACAGTGGATAA 207  
 Sbjct 20193 GTTCTGCACCTAACAGTGGATAA 20215

[Download](#) - [GenBank](#) [Graphics](#) [Next](#) [Previous](#) [Descriptions](#)

**Cloning vector H3.3K27M, complete sequence**  
 Sequence ID: [MH396437.1](#) Length: 18316 Number of Matches: 1

Range 1: 14844 to 14847 [Search](#) [Display](#)

Score	Expect	Identities	Gaps	Strand
322.981(174)	3e-84	194/203(95%)	4/203(1%)	Plus/Plus

Query 8 TTTAAAAGGGTATACAAAAGCC-AAATCTGGTACACAAAGGAATTTAGCGATGTTGG 86  
 Sbjct 14844 TTTAAAAGGGTATACAAAAGCC-AAATCTGGTACACAAAGGAATTTAGCGATGTTGG 14704

Query 67 AAAGGGTTTTATAGTACCCGAC-ATAAATACGACGCTGGGATACCTGGAGATGATG-A 124  
 Sbjct 14706 AAAGGGTTTTATAGTACCCGAC-ATAAATACGACGCTGGGATACCTGGAGATGATG-A 14704

Query 125 AACCCCTCTCTGGAAAAGCTGGAAAGCTGGTCAAGTGAAGTATCTGGACTGACGAA 184  
 Sbjct 14746 AACCCCTCTCTGGAAAAGCTGGAAAGCTGGTCAAGTGAAGTATCTGGACTGACGAA 14824

Query 185 GTTCTGCACCTAACAGTGGATAA 207  
 Sbjct 14825 GTTCTGCACCTAACAGTGGATAA 14847

### Appendix III: Reagent composition

#### Chemical pathology reagent concentration

- 1) Urea reagents:
  - R1- EDTA
  - R2- Phenol
  - R3- Sodium hypochlorite
  - Std Reagent- Calibration
- 2) Creatinine
  - R1- Sodium hydroxide (NaOH)
  - R2- Picric acid

© 2019 Rimi et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:  
 The peer review history for this paper can be accessed here:  
<http://www.sdiarticle4.com/review-history/52699>