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Application of Metagenomic Study for the Detection of Diphtheria Toxin Gene of *Corynebacterium diphtheriae* in Patients Displaying Symptoms of Pharyngitis in Kaduna State

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MIB and ABS designed the study, wrote the protocol and wrote the initial draft of the manuscript. Authors MSA and MIB performed the statistical analysis. Authors MIB, AAJ and YOAA managed the analyses of the study. Authors MIB and MB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This study was set to detect the A and B subunit of Diphtheria toxin gene among patients with pharyngitis and to identify which of the possible biovars is endemic in Kaduna State. The result of standard collection of twenty-two (22) throat swab samples of patients using standard technique of

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purposive sampling, packing in sterile water at 4°C and DNA extraction using the phenol/ chloroform extraction method gave two 2 (9%) positive PCR samples out of the twenty-two, (22) throat swabs analysed; both A and B subunits of the gene's amplicon were identified at 248bp and 297bp respectively. Sanger's (dye-terminator) sequencing of one of the genes' two, 2 subunits yielding two sequences obtained in the fasta format for bioinformatics analysis using the blast tool at the NCBI website. The 2 subunits of the gene were homologous to the biovars *gravis* and *mitis* complete coding sequence gene of *Corynebacterium diphtheriae* biovar *mitis* and *gravis* strains with percentage identity of 97 to both strains. Mutation in the 2 subunits of genes was also noticed with a total of 8 gaps that signify indels.

Keywords: Metagenomics; pharyngitis; Corynebacterium diphtheria; diphtheria; diphtheria toxin gene.

1. INTRODUCTION

1.1 Background of the Study

Metagenomics is the study of genetic material recovered directly from environmental samples. The broad field may also be referred to as environmental genomics, ecogenomics or community genomics. Traditional microbiology and microbial genome sequencing and genomics rely upon cultivated clonal cultures [1]. In biotechnology; microbial communities produce a vast array of biologically active chemicals that are used in competition and communication [2].

Pharyngitis is the inflammation of the pharynx, and it is the most common cause of sore throat which can be chronic or acute. It can be accompanied by a cough or fever, especially if caused by a systemic infection [3]. *Pharyngitis may be caused by Corynebacterium diphtheriae;* 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) [4].

Corynebacterium diphtheriae is a Gram-positive, non-spore forming, non-motile, pleomorphic rod belonging to the genus *Corynebacterium* and the actinomycete group of organisms causing the disease diphtheria. The organism produces a potent bacteriophage-encoded protein exotoxin, diphtheria toxin (DT), which causes the symptoms of diphtheria. This potentially fatal infectious disease is controlled in many developed countries by an effective immunisation programme with a vaccine called Diphtheria toxoid (DTaP) [3,5].

Unfortunately, in Nigeria, data and reports on Diphtheria have only been submitted 4 times in 14 years with the last report of 312 cases in 2006, 790cases in 2002 and 2,468 in 2001 [6] based on WHO report, diphtheria cases in India were 3380 cases in 2016.

Diphtheria has been a major silent killer disease almost all over the world and it is estimated that the disease is fatal in 5% and 10% of cases with children and adults above 40 years having a fatal rate of up to 20% [7]. This is caused by the toxigenic strains of *Corynebacterium* species. Currently, there are 4 known biovars [3] but unawareness about which of the strains present in Kaduna state poses a problem in managing it as the vaccine and anti-toxin are both produced from the tox genes which are specific to each biovar to make them more portent.

However, diphtheria became a medical rarity after the advent of vaccine [8]; it's screening, management, clinical clerking of patients, knowledge about the disease by the general public and administration of booster doses to increase immunity have been discontinued, taken lightly or forgotten by health practitioners especially in developing nations [8]. This is serious now that there is a striking resurgence of diphtheria in the Newly Independent State and sporadic occurrences across the globe which demands for urgent need of revival.

In Nigeria, the mortality rate of 33.3% in Benin City, Edo state [9] and a recent outbreak that claimed many lives in Kimba village, Borno State [10] have been recorded with poor management and lack of antitoxin to properly treat the patients.

Diphtheria can be considered as one of the neglected tropical diseases and therefore the need for its studies. Knowing the genes involved may give an opportunity for the

identification of the indigenous strain of the organism which could be used for the formulation of more effective vaccine to be administered to the general public. Again studies on the genes will enable detection of mutations either in the A or B subunit which is central for drug and vaccine production as the strain might be producing a different and identical exotoxin that could be resistant to the available treatment.

The best method for studying the Diphtheria toxin (DTx) gene is by metagenomic approaches; as culturing the throat swabs impairs with the gene isolation as demonstrated by Nakao and Popovic in 1997; 5 throat swabs out of 10 were negative from cultured *C. diptheriae* while only one was negative using metagenomic approaches. This was supported by Kobaidze [11] where swab collections of nose and throat specimens from patients with clinically defined diphtheria were PCR positive even after prolonged storage of 5-12months in gel packages at 4°C, while the culture as expected was negative. There is a death information on diphtheria in the Kaduna State which demands for studies of the organism.

As long as the disease continues to play a major role as a lethal resurgent infectious disease [12, 13], studies on Diphtheria from every angle is fundamental in managing it.

This study is aimed to detect and identify the Diphtheria Toxin gene (DTx) metagenomically using molecular techniques among patients with pharyngitis.

2. MATERIALS AND METHODS

2.1 Description of the Study Area

This study was conducted in Barau Dikko Specialist Hospital; Kaduna State, Nigeria. Kaduna State is located at Northwest of Nigeria; it covers a total area of 46,053 km^2 (17,781 sq mi) and an area rank of 4th of the 36 states of Nigeria. It has a population of 6,066,562 people going by 2006 census leaving it the 3rd of the 36 states of Nigeria in rank and a density of 130km^2 (340/sq mi) [14]. Its coordinates are 10^{o}20^{1}N and 7^{o}45^{1}E; these coordinates clearly indicate that the location is centralised and connects the major routes reaching most of the states of the Nation [14] and so when a communicable disease is endemic in the area it will pose as a threat to the nation as a whole. Its water supply is sourced through damping of rivers and digging of wells and boreholes [14] which places the town at a risk of counteracting the disease as it is also water Bourne. Kaduna State consists of twenty-three (23) Local Government Areas [14], a number quite large and ignorant on the infectious nature of diphtheria.

2.2 Sample Population

Subjects for this study included both adults and young patients with pharyngitis in Barau Dikko Specialist Hospital, Kaduna.

2.3 Sample Collection and Transportation

Clinical throat swabs were collected from patients with respiratory infections in Barau Dikko Specialist Hospital; Kaduna. These clinical samples were then transported to DNA laboratory and Molecular Research Institute in Sterile water at 4°C.

2.4 Bacterial Strains and Processing of Clinical Samples

2.4.1 Control

Water was used as the negative control.

2.4.2 Specimen collection and processing

A total of 22 samples of throat swabs were collected from patients with symptoms of pharyngitis using purposive sampling technique. Cotton wool swab sticks were used for the collection from the throat and were then packed in sterile water packages for transport to DNA laboratory Kaduna for further investigations according to the following methodology.

2.5 Isolation and Purification of DNA from the Swabs

This was achieved using the phenol/ chloroform extraction method described by Kirby in the year 1996 [15]. Using labelled Eppendorf tubes containing cells from the swab and sterile water; lysis buffer and proteinase K were then added. After vortexing and incubation at 65°C for 1 hr; phenol and chloroform were then added to harvest the DNA and purified using ethanol and its different concentration and temperatures following series of incubation.

2.6 Primer Reconstitution Stage

Primers were designed and procured by Bioneer Company. The tools involved are BLAST primer design tool specific for the alpha and beta subunits of the tox gene which were sent to the Bioneer company for procurement and the major steps include the primer being more than 20bp for stability and the melting temperature was the same with the template strand for smooth PCR operation.

2.7 Amplification of the Tox Gene (DT Gene)

This involved the use of 2 separate primers to initiate the making of several million copies of the tox gene from the long strand of DNA isolated and purified following changes in temperature to obtain DNA denaturation, primer ligation, polymerisation and annealing.

2.8 First PCR Reaction

This was performed in a programmable DNA Thermal Cycler PTC-100 with a total of 2ul of DNA solution and master mix of Accupower hot start PCR premix by Bioneer Company according to manufacturer's instructions with primers Tox1 and Tox2.

2.8.1 Second PCR reaction

The above procedure in the first PCR was applied to the sample DNA but the only difference was that the 2nd set of primers Diphth6F and Diphth6R for amplification of the beta subunit of the tox gene were used and annealing temperature of 48°C.

2.9 Visualisation of Amplicon

This was achieved using 1.5 g QDLE agarose gel dissolved in 100 ml of distilled water; formation of combs for reception of PCR products and Transilluminator for viewing under the UV light.

2.10 Sequencing Reaction

This was achieved using Dye Terminator Cycle Sequencing with Quick Start Kit, the first step was Ethanol Precipitation, then Sample Preparation for Loading in to the Instrument according to manufacturer's instructions.

2.11 Statistical Analysis

Data obtained from the manifestation and clinical symptoms along with demographic studies of patients were analysed using simple descriptive statistics involving frequency and percentages.

3. RESULTS

3.1 PCR Results

The PCR results are presented in Plates 1, 2 and 3. Plate 1 and 2 indicate 100% sensitive PCR results while plate 3 indicate 0% sensitive PCR results.

3.2 Sequencing Results

3.2.1 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:

ACAAAAGCCAAAATCTGGTACACAAGGAAAT
TATGACGATGATTGGAAAGGGTTTTATTATAT
ACCCAACAATAAATACGACGCTGCGGGATAC
TCTGTAGATAATGAAAACCCGCTCTCTGGAA
AAGCTGGAGGCGTGTGGTCAAAAGTGACGT
ATCCAGGACTGACGAAGGTTCTCGCACTAAA
AGT

Table 1. 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) (GAAAACTTTTCTTCGTACCA CGGGACTAA)	248bp for the A subunit and 297bp for the B subunit	Nakao and Popovic; 1997
	Set 2: Dipht 6F (PRN FORWARD) (ATACTTCCTGGTATCGGTAGC) and Dipht 6R (PRN REVERSE) (CGAATCTTCAACAGTGTTCCA)		

The polymerase chain reaction (PCR) amplification of the DT gene was conducted according to the method of Nakao and Popovic (1997) [16]

3.2.2 Sequence for the B subunit of the DTx gene

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

CCGCCACAATATCGACGGGACGATTATACGA
ATTATAGAACTACTTGAAATAAATTGATAATA
CTCTCTACAAAATTATATGCAGGCGAGACCA
ATATCGACTAGCTCTCCTACCAATGGAATAG
CTTGAGCAACCATTAAAGACGATAAAGCTATT
GATTGTGCCACTATCTCTTCTGTATTGTGGTG
AACGGCACCGTCTGCAATG

Table 2. Age distribution of patients according to years

Age group (years)	Frequency	Percentage (%)
0-9	5	22.7
10-19	2	9.1
20-29	8	36.3
30-39	2	9.1
40-49	1	4.5
AD	4	18.1

Total number of patients=22 with their frequency and percentage conversion*

3.3 Basic Local Alignment Search Tool (Blast) Results

These were results obtained by inserting sequences in the FASTA format in a search tool where the program compared nucleotide sequences of the A and B subunit of the Tox gene to sequence database and statistical significance of matches calculated.

4. DISCUSSION

Out of the 22 throat swabs screened using PCR; 2 (9%) of patients were 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 [17]. 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 [17]. 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 [18]. 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 [19,20]. 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 [10].

The two patients positive for diphtheria both took the vaccine since childhood according to their

Table 3. Demographic studies of patients

Diphtheria symptoms	Frequency	Percentage (%)
Gender	9 (F), 13 (M)	40.9 Females, 59 Males
Voice Change	8	36.3
Contact history with diphtheria case	0	0
Pharyngitis	22	100
Sore throat	10	45.4
Fever/ Headache	12	54.5
Cough	8	36.3
Nasal discharge	1	4.5
Nausea/ Vomiting	3	13.6
Diarrhoea	0	0
Otologic Symptoms	3	13.6
Dysphagia	10	45.4
Neck swelling	9	40.9
Enlarged tonsils	10	45.4
Membrane on Tonsils	0	0
Immunization status	15	68.1

The demographic studies of patients representing respective symptoms of diphtheria and percentage conversions

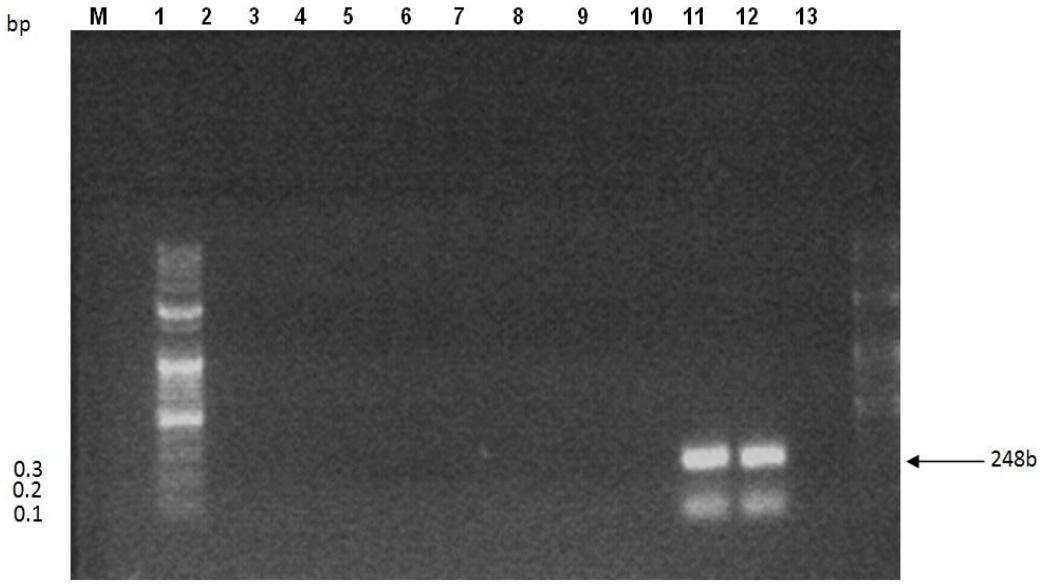

Plate 1. Shows 2 positive PCR results. Lanes 1 and 13- 10kb Moleculer weight marker, lane 1-8- 0% sensitive, lane 9- sample 10 and lane 10- sample 11 show two 100% sensitive PCR amplicons at 248bp for A subunit of the Diphtheria Tox gene

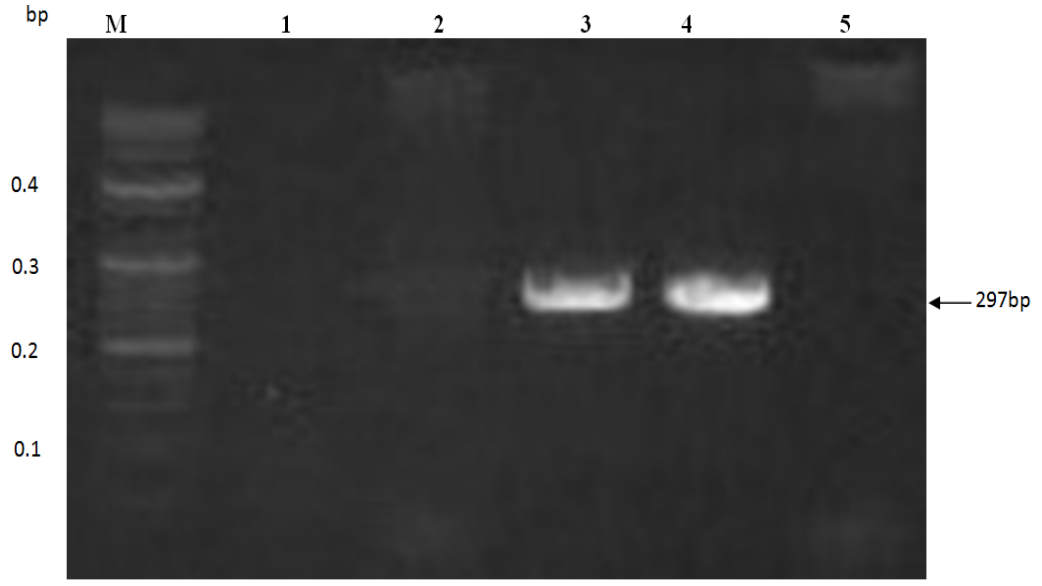

Plate 2. Shows 2 sensitive PCR results. M- 1kbp molecular weight marker, lane 1-negative control (PCR mix water), lanes 2 and 5- 0% sensitive sample, lane 3- sample 10 and lane 4- sample 11. Samples 10 and 11 show two 100% sensitive PCR amplicons at 297bp for B subunit of DTx gene

response and both were not up to date with the booster doses. In conformity; a case was seen in a 40-year-old man who acquired a cutaneous infection with *C. diphtheriae* var. *mitis* in Indonesia. The patient had been fully immunized in childhood and had received a booster prior to travelling in 1996; one of his household contacted the disease as she had a positive throat culture for *C. diphtheriae* [21]. This could be as a result of different strain prevalent in his destination that are resistant to the vaccine he took prior to travelling.

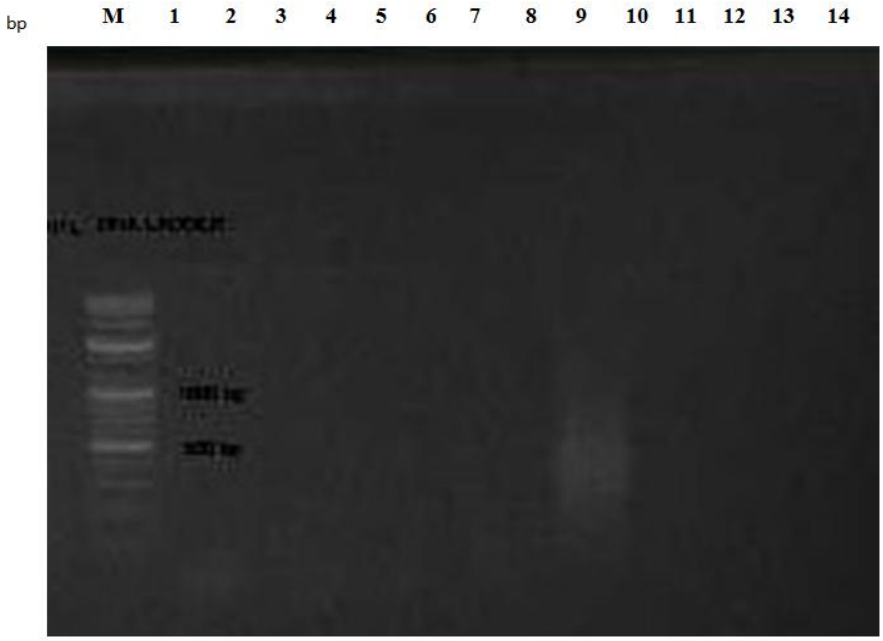

Plate 3. Showing 0% sensitivity of samples. M- 1kb molecular weight marker, lane 1-negative control, lanes 2-14- 0% sensitive PCR samples

Table 4. Summary of blast results

Dtx Gene	Bits	E-Value	1st Organism	2nd Organism	% Homology
A subunit	313	1e-81	*Corynebacterium diphtheriae* bv. *Gravis* strain 100-12 diphtheria toxin (tox) gene complete cds	*Corynebacterium diphtheriae* bv. *Mitis* strain 42-13 diphtheria toxin (tox) gene complete cds.	97 to both organisms
B subunit	340	5e-90	*Corynebacterium diphtheriae* bv. *Gravis* strain 100-12 diphtheria toxin (tox) gene complete cds	*Corynebacterium diphtheriae* bv. *Mitis* strain 76-11 diphtheria toxin (tox) gene complete cds.	97 to both organisms

The summary of blast results indicating the 2 subunits' bits which stands for number of matches, E-value for mutation detection, their matches and homologous percentage

The patients were also treated using antibiotics but the symptoms persisted even after administration and infusion with fluids. This is strongly correlated to the 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 [9].

In this study; BLAST results of the A and B subunits that showed the tox gene belonged to either the biovar *mitis* or *gravis* strain of the organism as it showed percentage identity of 97% for both the A and B subunit to the 2strains. 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 [10]*. 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 [22]. Similarly; in Canada; a toxigenic *C. diphtheriae* biovar *mitis* strain was recovered from a toe infection of a male patient that travelled to India [23]. Within the NIS; biotype var. *Gravis* and var. *Mitis* isolates were detected during different stages of the epidermis using

molecular analyses in the years 1984 through 1995 [6]. Lastly; *C. diphtheriae* var. *Mitis* was also isolated in a cutaneous infection in Indonesia [24].

A total of 8 gaps were noted which could signify mutation; 3 gaps in the A subunit and 5 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 [25].

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 1e-81 and 5e-90 respectively which are less than 1 [26]; it can be deduced that the tox gene isolated has mutated. This is also confirmed by the total sum of 8 gaps noticed from the blast results (3gaps from the A and 5gaps from the B subunit) signifying “indels” [26].

5. CONCLUSION AND RECOMMENDATIONS

The DTx gene was isolated in Kaduna State is homologous to the complete cds gene of *Corynebacterium diphtheria gravis* and *mitis* strains and there is mutation noticed in both the 2 subunits of the gene. From the findings and criteria used for sample collection; there are sporadic occurrences of diphtheria of either the *mitis* or *gravis* biotype strain in Kaduna state; Nigeria. Awareness should be made to the general public on the effect of diphtheria and ways to prevent it; awareness should also be made on the protocols to be followed in achieving full immunity against disease; especially the booster immunization that is supposed to be routinely taken every 10 years. The revival of routine screening of all throat swabs is ought to be done to patients presented with pharyngitis and/or other respiratory symptoms for detection of the organism using sophisticated technology; potent and viable reagents; Hoyle’s tellurite medium and Eleks’s test among others by well trained and skilled Laboratory Scientist. Since according to this research; 8 gaps were noticed which signify indels; further studies should be carried out to know the position and effect of the mutation on the exotoxin which may be detrimental to human beings or may render the conventional management and treatment less effective.

CONSENT

All authors declare that written informed consent was obtained from the study subjects for publication of this paper and accompanying images.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Hugenholz P, Goebel BM, Pace NR. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. Journal of Bacteriology. 1998;180(18):4765–74. PMC 107498. PMID 9733676.
2. Committee on Metagenomics: Challenges and functional applications, National Research Council. The New Science of Metagenomics: Revealing the Secrets of Our Microbial Planet. Washington, D.C.: The National Academies Press; 2007. ISBN 0-309-10676-1.
3. Marx A, John Q. Rosen's emergency medicine: Concepts and clinical practice (7th ed.). Philadelphia, Pennsylvania: Mosby/Elsevier. 2011;Chapter 30.
   ISBN 978-0-323-05472-0.
4. 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.
5. Acerra JR. "Pharyngitis”; 2010. Available:eMedicine.medscape.com
6. World Health Organization. Diphtheria; 2016.
   Available:http://www.who.int/immunization monitoring_surveillance/data
7. Atkinson W, Hamborsky J, McIntyre L, Wolfe S. Diphtheria. Epidemiology and prevention of vaccine-preventable

diseases (10 ed.). Public Health Foundation. 2007;59–70.
8. Long SS. Diphtheria. *Corynebacterium diphtheriae*. In: Behrman R.E., Kliegman R.M., Jensen H. B editors. Nelson textbook of Pediatrics. Philadelphia WB Saunders Company. 2000;817-820.
9. 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 1595-689X.
10. Besa NC, Coldiron ME, Bakari A, Raji A, Nsuami MJ, Rousseau C, Hurtado N, Porten K. Article of diphtheria outbreak with high mortality in Northeastern Nigeria; 2011.
11. Kobaidze K, Popovic T, Nakao H, Quick L. Direct polymerase chain reaction for *Corynebacterium diphtheriae* strains from the Republic of Georgia after prolonged storage. Journal of Infectious Disease 2000;181(suppl 1):S152-5.
12. Galazka AM, Robertson SE. Diphtheria: Changing patterns in the developing world. Europe Journal Epidemiology. 1995; 11:107-17.
13. Dittman S. Epidemic diphtheria in the Newly Independent States of the former—situation and lessons learned. Textbook of Biology Logicals. 1997;25:179-86.
14. Available:www.maplandia.com/kaduna/dokakaw
15. Kirby KS. Isolation of nucleic acids with phenolic solvents. In methods in enzymology. (Grossman, L and Moldave, K., Eds). Academic Press New York. 1968; XIIB:87-100.
16. Nakao H, Popovic T. Development of a direct PCR for detection of the diphtheria toxin gene. Journal of Clinical Microbiology. 1997;35:1651-5.
17. Public Health England. Infection report/ immunization. Diphtheria in England and Wales; Health Protection Weekly Report. 2016;10(13).
18. Mikhailovich VM, Melnikov MG, Mazuroval K. 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.
19. Pallen MJ, Hay AJ, Puckey LH, Efstratiou A. Polymerase chain reaction for screening clinicalical isolates of corynebacteria for the production of diphtheria toxin. Journal of Clinical Pathology. 1994;47:353-6.
20. 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.
21. Efstratiou A, George RC. Microbiology and epidemiology of diphtheria. Textbook of Revised Medical Microbiology. 1996; 7:31–42.
22. Public Health Laboratory Service. Diphtheria acquired during a cruise in the Baltic Sea. Communicable Disease Representative CDR Weekly. 1997;7:207.
23. Snyder JW. Media for detection of *Corynebacterium diphtheriae,* In GarciaL. S. *(ed.),* Clinical microbiological procedures handbook, 3rd ed., ASM Press, Washington DC. 2010;1:3.11.7.
24. Engler KH, Glushkevich T, Mazuroval K, 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.
25. 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. 2000;181: S163.
26. Jin X. Pairwise sequence alignment. Textbook of Essential Bioinformatics, Texas A & M University, Cambridge University Press. 2006;38 and 48.
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