Original Article

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Molecular Characterisation of *Corynebacterium diphtheriae* Isolates of Faucial Diphtheria Cases from Assam: A Cross-sectional Study

Microbiology Section

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ABSTRACT

Introduction: Diphtheria caused by toxigenic *Corynebacterium diphtheriae* is an acute respiratory infection characterised by pseudomembrane formation in the throat. Although diphtheria is often reported sporadically across India, molecular characterisation of *C.diphtheriae* strains could play a crucial role in epidemiological investigations.

Aim: To perform molecular characterisation of *Corynebacterium diphtheriae* isolates of faucial diphtheria cases from Assam, North-East India utilising Multi-Locus Sequence Typing (MLST).

Materials and Methods: In this cross-sectional study, three isolates were obtained from three different cluster of cases that occurred across two districts of Assam, India for a period from November 2019 to June 2020. The MLST of these three *C.diphtheriae* isolates were performed in Multidisciplinary Research Unit of Assam Medical College and Hospital by using the 7-gene MLST alleles of *C.diphtheriae* to determine the Sequence Types (ST).

Results: The MLST of the three isolates of this study revealed two distinct STs, ST-588 and ST-576 with multi-clonal lineages with ST-466, reported in *C.diphtheriae* strains of North Kerala and ST-301 related strains of Bangladesh, respectively.

Conclusion: In spite of frequent reports of diphtheria cases from Assam, molecular typing of the *C.diphtheriae* strains found in North-Eastern region of India has not yet been performed. This is one of the initial investigations from this region of India to use MLST for typing *C.diphtheriae* strains. Therefore, studies related to molecular characterisation of *C.diphtheriae* strains found throughout India could immensely help in determining the clonality and diversity existing among these strains.

Keywords: Clonal distribution, Diphtheria, Epidemiological investigation, Multi-locus sequence typing, Phylogenetic tree

INTRODUCTION

Diphtheria caused by *Corynebacterium diphtheriae* is an acute infectious disease ranging from mild infection to acute airway obstruction, toxaemia and sudden death [1]. The disease can have a fatality rate as high as 30.8% as reported in a previous study from Assam [2]. This disease is not only prevalent in Assam, a North-Eastern state of India, but also in other parts of India [3]. However, due to widespread immunisation coverage in children, a shift in burden of diphtheria cases has been observed in adolescents and adults [4]. However, cases are still reported in children of marginalised population [5].

Faucial diphtheria is characterised by the formation of a pseudo-membrane on one or both tonsils that extends to the tonsillar pillars, uvula, soft palate, oropharynx, and nasopharynx. Further extension of the pseudo-membrane into the larynx or trachea or dislodgement of a piece of it may even lead to fatal airway obstruction [6].

The causative agent, *Corynebacterium diphtheriae*, isolated from diphtheria cases needs to be monitored and characterised to know the prevailing ST, to be able to detect any evolution in the bacteria and contain any possible spread of Corynebacterium diphtheria strains. Various reports on diphtheria cases from Assam have been published [2,7-9]. However, studies on molecular typing of *C.diphtheriae* using techniques like RFLP, MLST, etc., have not been done from the North-East part of India. Hence, this study was done to utilise the 7-gene MLST alleles of *C.diphtheriae* to determine the ST and illustrate the epidemiology of the strains prevalent in this endemic region.

MATERIALS AND METHODS

A laboratory based cross-sectional study was undertaken in the Multi-disciplinary Research Unit of Assam Medical College and Hospital, Dibrugarh during the period from November 2019 to June 2020.This study was approved by the Institutional Ethics Committee of Assam Medical College and Hospital, Dibrugarh (IEC No. AMC/EC/185; Reg.no. ECR/636/Inst/AS/2014).

Inclusion criteria: *Corynebacterium diphtheriae* isolated from index cases of diphtheria outbreaks was included in the study.

Exclusion criteria: *Corynebacterium diphtheriae* isolated from cases other than the index cases of diphtheria outbreaks was excluded from the study.

Study Procedure

Three *C.diphtheriae* isolates, isolated during the period from 2017 to 2019 from index cases of diphtheria outbreaks which occurred in Jorhat and Dibrugarh districts of Assam were subjected to MLST. Among these isolates, two isolates (AMC1 and AMC2) were isolated in the Department of Microbiology, Assam Medical College and Hospital, Dibrugarh and one isolate (JMC1) isolated in Department of Microbiology, Jorhat Medical College and Hospital, Jorhat. Further molecular characterisation of these isolates was performed in the Multi-disciplinary Research Unit of Assam Medical College and Hospital. These isolates were confirmed as *C.diphtheriae* by standard biochemical methods and PCR amplification of *rpoB* and *toxA* gene of *C.diphtheriae*.

Bacterial DNA extraction: Bacterial DNA was extracted using QIAamp DNA mini kit (Qiagen Inc., USA) following manufacturer's instructions and the extracted DNA was quantified using the µDrop plate in Multiskan GO (Thermo Scientific, USA).

PCR identification of *C.diphtheriae* isolates: The PCR targets of *rpoB* gene of *C.diphtheriae*, *C. ulcerans*, and *toxA* gene fragment of *C.diphtheriae* were used for identification of the *C.diphtheriae* isolates. The PCR amplification was carried out in a 25 µL reaction volume consisting of 12.5 µL of 2X Bioline MyTaq HS Mix (Meridian Biosciences, USA) and 500 nM of each forward and reverse primers.

The PCR conditions consisted of an initial denaturation of 95°C for 1 minute followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 10 seconds. The PCR amplicons were visualised for respective PCR bands on a 2% agarose gel stained with ethidium bromide [7].

Multi-Locus Sequence Typing (MLST): The MLST for *C.diphtheriae* isolates were performed by PCR amplification and sequencing of the seven housekeeping genes (*atpA, dnaE, dnaK, fusA, leuA, odhA* and *rpoB*) as per the protocol described by Bolt F et al., [10]. Purification of the amplified PCR products was performed by using ExoSAP-IT PCR product clean-up reagent (ThermoFisher Scientific, USA) by following manufacturer's instructions. Cycle sequencing was performed by using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and sequencing was performed using Genetic Analyser 3500 (Applied Biosystems, USA). Consensus FASTA sequences were generated in BioEdit sequence alignment editor (Version 7.2.5) by multiple sequence alignment of both forward and reverse reads [11].

The population snapshot analysis of the 1357 isolates available till date in the *C.diphtheriae* MLST database was performed by utilising goeBURST algorithm in Phyloviz (Version 2.0) [12]. The phylogenetic tree was constructed using the concatenated allele sequences in MEGA (Version X) using Neighbour-Joining method and Jukes Cantor algorithm. The test of phylogeny was done with 1000 bootstrap replicates [13]. The allele sequences of the isolates of this study were submitted to *C. diptheriae* and *C.ulcerans* MLST sequence database (http://pubmlst.org/cdiphtheriae) and STs were assigned to each of the three isolates of this study. The isolates AMC1, AMC2, and JMC1 have been submitted to *C. diptheriae* and *C. ulcerans* MLST isolate database with isolate ids 851, 852, and 853 respectively [14].

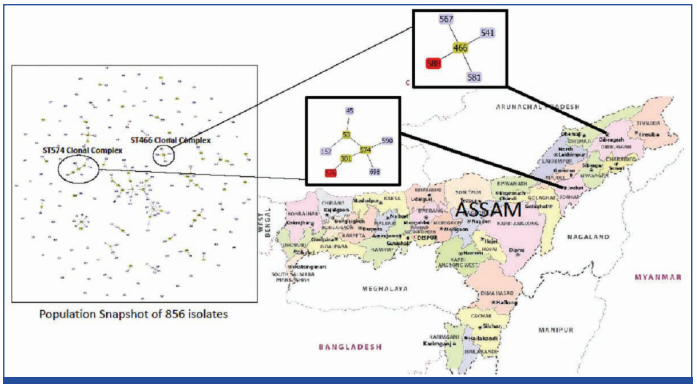
STATISTICAL ANALYSIS

As this study involves only three isolates of *C. diphtheriae*, statistical methods could not be applied.

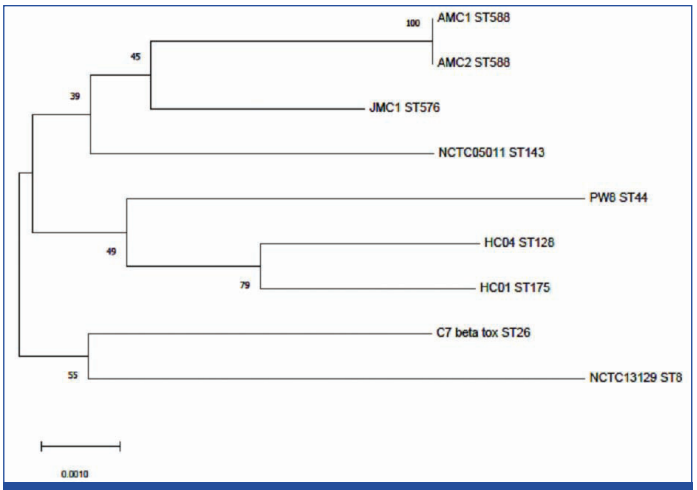
RESULTS

Population snapshot and phylogenetic analysis: Total three *C. diptheriae* isolates from each index case of three different diphtheria outbreaks were studied for MLST. The allele sequences of these isolates of this study were submitted to the *C. diptheriae* MLST sequence database (https://bigsdb.pasteur.fr/diphtheria/) and STs were assigned to each of the three isolates of this study. The isolates AMC1, AMC2, and JMC1 have been submitted to *C. diptheriae* MLST isolate database with isolate ids 2113, 2114, and 2115, respectively.

The population snapshot analysis of the 856 isolates available till date in the *C. diptheriae* MLST database revealed that the isolates AMC1 and AMC2 bearing ST-588 was assigned to the ST-466 Clonal Complex (CC) and is a Single Locus Variant (SLV) of ST-466 [Table/Fig-1]. The JMC1 isolate with ST-576 was assigned to the ST-574 CC and is a SLV of ST301 [Table/Fig-1]. The phylogenetic tree constructed using the concatenated allele sequences of seven housekeeping genes of the three *C.diphtheriae* isolates of this study along with reference strains (NCTC 05011, HCO1, HC04, C7betatox+, NCTC 13129, and INCA402) obtained from different parts of the world [Table/Fig-2]. The isolates AMC1, AMC2, and JMC1 of this study were found to be in close relation with the NCTC 05011 strain with ST-143 originating form United Kingdom. None of the three isolates of this study were related to toxigenic reference strains of C7 beta tox (ST-26) and NCTC 13129 (ST-8).



[Table/Fig-1]: Population snapshot analysis of all the STs available till date in the *C.diphtheriae* MLST database is illustrated in the larger square box. The clonal complexes 466 and 574 are illustrated as magnified images of the population snapshot, STs 576 and 588 highlighted in red are from this study. The magnified images are also pointing towards their geographical location of isolation.



[Table/Fig-2]: Neighbour-joining tree constructed using the concatenated allele sequences of the seven *C.diphtheriae* housekeeping genes of three *C.diphtheriae* isolates of this study along with reference *C.diphtheriae* strains (NCTC 05011, HCO1, HC04, C7 betatox+, NCTC 13129, and INCA402) isolated from various parts of the world. The isolates AMC1, AMC2, and JMC1 are form this study.

DISCUSSION

Diphtheria owing to its high mortality rate was a much-feared disease before the introduction of a mass immunisation programme in India. However, there have been resurgences of this infectious disease in many parts of the country despite the high immunisation coverage. Outbreaks and sporadic cases of diphtheria have been reported occasionally from Assam [Table/Fig-3] [2,7-9,15]. In one of the previous studies from Assam, 33 cases were reported and 10 cases were confirmed as *C.diphtheriae* by culture [7]. The MLST plays a crucial role in epidemiological investigation of pathogens. In 2016, *C.diphtheriae* strains with ST-466 were isolated during a national program surveillance in North Kerala. These ST-466 strains were found to be toxigenic as confirmed by Modified Elek's Test [3]. The isolates AMC1 and AMC2 of this study with ST-588 is a SLV of ST-446 and were found to be positive for the *toxA* gene as confirmed by PCR. During December 2017, an outbreak among the Forcibly Displaced Myanmar Nationals (FDMN) in Bangladesh occurred, in response to which Centers for Disease Control and Prevention (CDC) provided technical aid to study and characterise the *C.diphtheriae* isolates. This reported outbreak included three novel STs, two of which were also closely related to ST-301 [16]. The

Author and reference	Year of study	District	Setting	Total cases
Nandi R et al., [9]	1997-2002	Silchar	Hospital	101
Nath B and Mahanta TG [8]	2009	Dibrugarh	Outbreak	60
Saikia L et al., [2]	2010	Dibrugarh	Outbreak	13
Das PP et al., [7]	2015-2016	Dibrugarh	Outbreak	33
Devi U et al., [15]	2017	Dibrugarh	Surveillance	164
Choudhury G et al., (Present study)	2019-2020	Dibrugarh, Jorhat	Outbreak	3

[Table/Fig-3]: Diphtheria cases reported from various parts of Assam [2,7-9,15].

ST-576 was assigned JMC1 isolate of this study is a SLV of ST301. The Indian state of Assam shares an international border of 263 kilometres with Bangladesh which may explain this clonality existing among the *C.diphtheriae* isolates of Assam and Bangladesh.

The MLST database of *C.diphtheriae* includes a total 1357 isolate data submitted till date from various parts of the world [14]. However, only thirteen isolate data including the three isolates reported in this study have been submitted to the database from India. Though sporadic cases have been reported frequently all over the country and a nationwide surveillance programme is still going on under Integrated Disease Surveillance Programme (IDSP), molecular typing of the isolates has not been performed in most of the cases [17].

Limitation(s)

This study only included three *C.diphtheriae* isolates from index cases from three different diphtheria outbreaks. However, a greater number of isolates are required to determine the prevalent STs in this region. Moreover, Elek's Gel precipitation test to determine the toxigenicity of these isolates was not performed in this study.

CONCLUSION(S)

Two distinct STs, ST-588 and ST-576 with multi-clonal lineages with ST-466, reported in *C.diphtheriae* strains of North Kerala and ST-301 related strains of Bangladesh respectively, were revealed. In spite of frequent reports of diphtheria cases from Assam, molecular typing of the *C.diphtheriae* strains found in this region has not yet been performed. This is one of the initial investigations from the North-Eastern region of India to use MLST for typing *C.diphtheriae* strains found in this region. More studies from entire India on ST of *C.diphtheriae* strains could help immensely on establishing the prevalent STs; clonality and diversity existing among the *C.diphtheriae* strains found in India. The MLST on a large number of isolates from various regions of North East India could be a future perspective. Also, the determination of the toxigenic potential of all outbreak isolates of the regions could be another scope for future research. Moreover, expanded diphtheria surveillance including other anatomical sites (cutaneous, mucosal) etc., could be placed for early management of the cases.

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REFERENCES

[1] Magill AJ, Ryan ET, Solomon T, Hill DR. Hunter's Tropical Medicine and Emerging Infectious Disease: Ninth Edition. Hunter's Tropical Medicine and Emerging Infectious Disease: Ninth Edition. 2012. 1-1190 p.
[2] Saikia L, Nath R, Saikia NJ, Choudhury G, Sarkar M. A diphtheria outbreak in Assam, India. Southeast Asian J Trop Med Public Health. 2010;41(3):647-52.
[3] Sangal L, Joshi S, Anandan S, Balaji V, Johnson J, Satapathy A, et al. Resurgence of Diphtheria in North Kerala, India, 2016: Laboratory Supported Case-Based Surveillance Outcomes. Front Public Health. 2017;5:218.
[4] Garib Z, Danovaro-Holliday MC, Tavarez Y, Leal I, Pedreira C. Diphtheria in the Dominican Republic: Reduction of cases following a large outbreak. Rev Panam Salud Publica. 2015;38(4):292-99.
[5] Wagner KS, White JM, Lucenko I, Mercer D, Crowcroft NS, Neal S, et al. Diphtheria in the postepidemic period, Europe, 2000-2009. Emerg Infect Dis. 2012;18(2):217-25.
[6] Hadfield TL, McEvoy P, Polotsky Y, Tzinserling VA, Yakovlev AA. The Pathology of Diphtheria. J Infect Dis. 2000;181(Suppl 1):S116-20.
[7] Das PP, Patgiri SJ, Saikia L, Paul D. Recent outbreaks of diphtheria in Dibrugarh District, Assam, India. J Clin Diagnostic Res. 2016;10(7):DR01-03.
[8] Nath B, Mahanta TG. Investigation of an outbreak of diphtheria in Borborooah block of Dibrugarh district, Assam. Indian J Community Med. 2010;35(3):436-38.
[9] Nandi R, Mriganka De, Simon Browning. Diphtheria: The patch remains. J Laryngol Otol. 2003;117(10):807-10.
[10] Bolt F, Cassiday P, Tondella ML, DeZoysa A, Efstratiou A, Sing A, et al. Multilocus sequence typing identifies evidence for recombination and two distinct lineages of Corynebacterium diphtheriae. J Clin Microbiol. 2010;48(11):4177-85.
[11] Hall T. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In Nucleic Acids Symp. Ser. 1999 (Vol. 41, pp. 95-98). Available from: https://www.academia.edu/2034992/BioEdit_a_user_friendly_biological_sequence_alignment_editor_and_analysis_program_for_Windows_95_98_NT.
[12] Nascimento M, Sousa A, Ramirez M, Francisco AP, Carriço JA, Vaz C. PHYLOViZ 2.0: Providing scalable data integration and visualisation for multiple phylogenetic inference methods. Bioinformatics. 2017;33(1):128-29.
[13] Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35(6):1547-49.
[14] BIGSdb-Pasteur. Institut Pasteur MLST databases and software [Internet]. [cited 2022 Jan 16]. Available from: https://bigsdb.pasteur.fr/diphtheria/.
[15] Devi U, Baruah PJ, Borah PK, Mahanta J, Dutta P. Report of diphtheria cases & surveillance among contacts in Dibrugarh, Assam, India. Indian Journal of Medical Research. Wolters Kluwer-Medknow Publications. 2017;145(6):847-48.
[16] Akram A, Laurence MG, Williams M, Weil L, Tahmina S, Muraduzzaman AKM, et al. (2019). Population Structure of a Large Diphtheria Outbreak Among Forcibly Displaced Myanmar Nationals- Bangladesh, 2017-2018. Available from: https://www.researchgate.net/publication/335203377_Population_Structure_of_a_Large_Diphtheria_Outbreak_Among_Forcibly_Displaced_Myanmar_Nationals_-Bangladesh_2017-2018.
[17] ToT Training Manuals For State/District Surveillance Officers, State RRTs & Nodal Officers From Medical Colleges. Integrated Disease Surveillance Programme: Ministry of Health and Family Welfare, Government of India, India. Revised- 2020. Available from: https://idsp.nic.in/WriteReadData/OldSite/TOTmanualSSO.pdf.

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