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Detection of *Clostridium tetani* in human clinical samples using *tetX* specific primers targeting the neurotoxin[☆]



Madhu Ganesh^a, Nasira K. Sheikh^a, Pooja Shah^a,
Gajanan Mehetre^b, Mahesh S. Dharne^b,
Basavraj S. Nagoba^{c,*}

^a Dr. VM Medical College, Solapur 413 001, India

^b NCIM Resource Centre, CSIR – National Chemical Laboratory (NCL), Pune 411 008, Maharashtra, India

^c Maharashtra Institute of Medical Sciences & Research, Latur 413 531, Maharashtra, India

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KEYWORDS

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Summary Tetanus resulting from ear injury remains an important health problem, particularly in the developing world. We report the successful detection of *Clostridium tetani* using *tetX* specific primers targeting the *Cl. tetani* neurotoxin. The sample was obtained from an ear discharge of a case of otogenic tetanus in a 2-year-old male child. Based on the culture results of the ear discharge, Gram staining and virulence testing by genotyping, a diagnosis of tetanus was confirmed. This is the first report from India on the successful detection of *Cl. tetani* in a human clinical sample using *tetX* specific primers targeting the *Cl. tetani* neurotoxin. © 2015 King Saud Bin Abdulaziz University for Health Sciences. Published by Elsevier Limited. All rights reserved.

Introduction

Tetanus is a potentially fatal muscle spasm disease caused by *Clostridium tetani*, a motile, spore-forming, anaerobic, Gram-positive bacillus. Although tetanus resulting from ear injury is extremely rare, it remains a major problem.

[☆] The GenBank/EMBL/DDBJ accession number for the *tetX* gene sequence of *Clostridium tetani* determined in this study is KM677991.

* Corresponding author. Tel.: +91 2382227424; fax: +91 2382227246; mobile: +91 9423075786/7588237531.

E-mail addresses: dr.bsnagoba@yahoo.com, bsnagoba@gmail.com (B.S. Nagoba).

Although it is less common in the developed world [1], it is a significant disease in the developing world. Despite a widespread immunization program, a large number of cases have been reported from the developing world [2–5]. The most common cause of otogenic tetanus is trauma followed by contamination of the wound [6]. Here, we report the isolation of *Cl. tetani* from a case of otogenic tetanus and its confirmation by culture and sequencing based detection and genotyping. To the best of our knowledge, this is the first report from India on the successful detection of *Cl. tetani* in a human clinical sample using *tetX* specific primers targeting the *Cl. tetani* neurotoxin.

Subject and methods

This study reports the microbiological analysis of a clinically diagnosed case of otogenic tetanus for confirmation of the clinical diagnosis and to confirm the virulence of the causative agent and to differentiate it from morphologically similar Clostridia.

A 2-year-old-non-immunized-male child presented with a history of fever for 4 days, trismus and constant cry, watery, non-foul smelling discharge from the ear for 6 days, gradually progressing to breathlessness, and progressively increasing to coughing and sneezing. Inability to open mouth, particularly after coughing and sneezing, and dysphagia for solid food, were reported by the mother.

Physical examination revealed tautness of the neck muscles with no neck swelling, trismus grade 4 and risus sardonius. The patient had an opisthotonus posture and a history of trauma to the left ear due to matchstick insertion 6 days prior. His pulse rate was 180/min, respiratory rate was 28/min and oxygen saturation was 94%.

The results of the routine investigations showed hemoglobin 9.4 g/dL, total leukocyte count of 13,200/mm³ with a monocyte count of 8%, platelet count of 367,000/mm³ and RBC count of 2.7 million/mm³. The random blood glucose was 50 mg/dL, urea was 21 mg/dL, creatinine was 0.8 mg/dL, serum sodium was 142 meq/L, potassium was 4.2 meq/L and calcium was 8.7 meq/L.

Microbiological analysis

Ear discharge was collected in Robertson's cooked meat (RCM) broth and processed for aerobic and anaerobic bacteriological analysis. A smear of RCM broth was prepared and stained with Gram stain. A loopful of RCM broth was inoculated onto freshly prepared blood agar and incubated aerobically and anaerobically in a McIntosh and Fildes jar. RCM

broth was incubated further at 37 °C to study proteolytic activity [7].

Sequencing based detection and genotyping of *Clostridium tetani* directly from clinical sample

DNA extraction

The ear discharge from the patient collected in RCM broth (stored at 4 °C) was used for DNA extraction. The total nucleic acid extracted from 1 mL of sample using a bacterial DNA kit (Merck-Genei) was resuspended in elution buffer. The quality of the extracted DNA was assessed by 0.7% agarose horizontal gel electrophoresis in TAE buffer (40 mM Tris, 20 mM acetate, and 2 mM EDTA) and visualized by GelRed™ staining on a Protein Simple gel documentation unit. The concentration of the extracted DNA was ascertained on a NanoDrop Lite spectrophotometer (NanoDrop Biotechnologies).

PCR amplification and sequencing

The *Cl. tetani* specific primers targeting a fragment of the *tetX* gene (1354 bp) were used to amplify the DNA extracted from the sample [8]. The PCR reaction contained 10 nM each primer (Eurofins), 200 μM each deoxynucleoside triphosphate (dNTP) (Genei), 1 U Taq polymerase (Genei) in the appropriate reaction buffer, and 50 ng and 100 ng of DNA extracts as the templates. The reactions were performed in a 50 μL reaction mixture. The cycling conditions were as follows: initial denaturation of 95 °C for 10 min, followed by 25 cycles each of 1 min at 94 °C, 1 min at 52 °C and 1.5 min at 72 °C. Positive PCR amplicons were documented followed by purification using the Exo-rSAP (New England Biolabs) method and sequenced on both strands in an ABI 3500xl genetic analyzer (ThermoFisher).

Sequence analysis

The obtained sequences were assembled and edited using the sequence analysis software version 5.1 (ThermoFisher). Edited sequences were submitted to BLASTN and BLASTX using the default parameters for analysis [9], followed by comparison with the closest homologous sequences retrieved from the GenBank database.

Based on the results of the microscopy, culture and molecular studies, a clinical diagnosis of otogenic tetanus was confirmed and the patient was given tetanus toxoid 0.5 mL IM, tetanus globulin 500 IU IM, crystalline penicillin 2.5 lac IV QID, metronidazole 20 mg/kg/day, and diazepam 1.5 mg TDS for 7 days along with oxygen by mask. The child recovered well and was discharged on the 8th day.



Figure 1 Gram stain smear showing characteristic drum stick appearance on 3rd day.

Results and discussion

Microscopic examination of the RCM broth using Gram staining showed Gram-positive bacilli with round terminal bulging spores (Fig. 1). Further incubation of the RCM broth showed proteolytic activity, and anaerobic cultures on freshly prepared blood agar showed a thin transparent film of growth with swarming typical of *Cl. tetani* (Fig. 2). Aerobic culture of the ear discharge showed no significant findings.

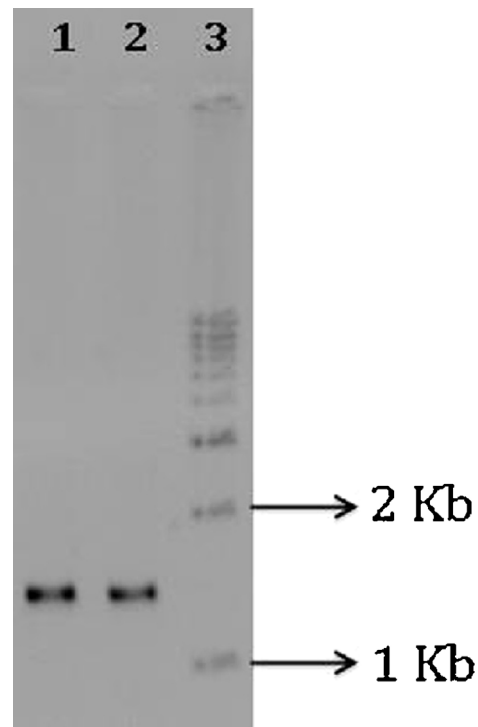


Figure 3 Polymerase chain based amplification of ear discharge using *tetX* specific primers. (Amplicons electrophoresed in an 0.8% agarose gel. Lane 1: 50 ng DNA from ear discharge, Lane 2: 100 ng DNA from ear discharge, and Lane 3: 1 Kb DNA ladder (Genei).)

The *tetX* gene fragment specific for the *Cl. tetani* neurotoxin was successfully amplified (approximately 1354bp) by PCR (Fig. 3) from 50 ng of template DNA. Although we obtained 415 bp sequences toward the 5' end, reverse primer failed



A



B

Figure 2 (A) Robertson cooked meat broth showing proteolytic activity. (B) Blood agar showing swarming.

Table 1 Homologies of sequences obtained from the ear discharge for the NCBI BLASTN and BLASTX databases.

Primer	BLASTN		BLASTX		Reference
	First match	Percent homology	First match	Percent homology	
Tet2F ^a	AJ891297 <i>Clostridium</i> spp. RKD partial gene for neurotoxin heavy chain 1	100% (415/415)	CAJ30283 Neurotoxin heavy chain 1 ^b <i>Clostridium</i> neurotoxin, N-terminal receptor binding; pfam07953	100% (122/122)	Deposited in the GenBank but not published
Primer	BLASTN		BLASTX		Reference
	Second match	Percent homology	Second match	Percent homology	
Tet2F	AM076940 <i>Clostridium</i> spp. RKD partial <i>tetX</i> gene for tetanus toxin precursor	100% (415/415)	CAJ28911 Tetanus toxin precursor/ tetanus toxin light chain ^b	100% (122/122)	Dixit et al. [10]

^a Binds to 5' end of *tetX* gene.

^b *Clostridium* neurotoxin, N-terminal receptor binding; pfam07953.

to yield sequencing data after repeated attempts. The sequence was compared independently for homology using BLASTN and BLASTX (Table 1). The sequence analysis displayed 100% homologies at the nucleotide and amino acid level with the *Cl. tetani* RKD strain (Indian origin) neurotoxin heavy chain 1 and light chain [10]. The *tetX* gene sequence obtained in this study was deposited in the GenBank under accession number KM677991 (Fig. 3).

Attempts to detect the tetanospasmin gene for the rapid definitive diagnosis of tetanus using the primer pairs GAT1 and GAT2 for S1, and GAT 5 and GAT6 for S2 were previously reported [11]. In the present study, tetanospasmin was detected using *tetX* specific markers from a case of otogenic tetanus.

Tetanus is a preventable and potentially fatal, muscle spasm disease caused by *Cl. tetani*. Trauma followed by contamination of the wound is the most common cause. Vaccination is the only way to prevent infection because infection by *Cl. tetani* does not confer immunity. Since the introduction of the vaccination program in 1961, there has been a significant decline in the number of cases in the western world [12]. Because of the increased coverage of immunization, the incidence has also decreased significantly from the developing world, but isolated cases have been reported from developing countries, including India [2–5].

Because of the significant decline following mass immunization, the occurrence of tetanus is overlooked, but the occurrence of isolated cases indicates that this disease remains. In particular, non-immunized and inadequately immunized individuals carry higher risks, and vaccination in pregnant women and children should be emphasized.

The diagnosis of tetanus is based on typical clinical features. Laboratory methods are used to confirm the clinical diagnosis and to rule out conditions, such as strychnine poisoning and dystonic reactions to antidopaminergic drugs, which mimic generalized tetanus [13]. PCR for the detection of neurotoxin not only helps in the rapid definitive diagnosis of tetanus but also helps in the confirmation of virulence and to rapidly differentiate *Cl. tetani* from other morphologically similar non-pathogenic Clostridia, including *Cl. tetanomorphum* and *Cl. sphenoides*. PCR studies are also helpful for the diagnosis of tetanus in patients with atypical symptoms, particularly for the confirmation of cases of otogenic tetanus with atypical clinical presentation.

This case emphasizes the need for a high index of suspicion of otogenic tetanus in a patient presenting with typical clinical features and no history of immunization. The presence of Gram-positive bacilli with round terminal spores, proteolytic activity and an anaerobic culture showing swarming

growth are important for confirming the diagnosis. DNA extraction, PCR amplification and sequencing of the *tetX* gene fragment specific for the *Cl. tetani* neurotoxin is important for confirming virulence.

Conflict of interest

Funding: No funding sources.

Competing interests: None declared.

Ethical approval: Not required.

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