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CASE REPORT

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

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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.

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.

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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 sardonicus. 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/mm^3 with a monocyte count of 8%, platelet count of 367,000/mm^3 and RBC count of 2.7 million/mm^3. 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 Filde's 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^{TM} 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.