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Full Length Research Paper

Detection of *Clostridium tetani* by fluorescent amplification based specific hybridization

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The etiologic agent of tetanus, *Clostridium tetani*, is an obligate anaerobic and spore-forming bacillus; and remains an important public health problem in developing countries. Although, the diagnosis is based mainly on history and presentation of tetanus symptoms. Some recent reports indicate that a patient may not manifest any symptoms of localized or generalized tetanus. However, one of the new approaches for accurate and rapid detection of microbial pathogens is a polymerase chain reaction (PCR) followed by fluorescence detection. In this report, we have described a fluorescent amplification based specific hybridization (FLASH) based PCR assay for detection of *C. tetani*. The results of the present study indicated that the FLASH based PCR assay appear to be a suitable and rapid method for identification of cultured isolates of *C. tetani*.

Key words: *Clostridium tetani*, molecular diagnosis, fluorescent amplification based specific hybridization (FLASH) polymerase chain reaction (PCR), fluorescent probe.

INTRODUCTION

The etiologic agent of tetanus, *Clostridium tetani*, is an obligate anaerobic, Gram-positive, rod-shaped and spore-forming bacillus; remains an important public health problem in developing countries (Poudel et al., 2009; Brook, 2008). Tetanus is now an important cause of death worldwide and is associated with a high case fatality, particularly in the developing world Poudel et al. (2009). The World Health Organization estimates that 59,000 newborns worldwide died in 2008 as a result of neonatal tetanus (http://www.who.int/en/). In recent years, approximately 11% of reported tetanus cases have been fatal (Sheffield and Ramin, 2004).

The disease is caused by the action of a neurotoxin, produced by the bacteria when they grow in the absence of oxygen, for example, in dirty wounds (WHO, http://www.who.int/topics/tetanus/en). The diagnosis is based mainly on history and presentation of tetanus symptoms (Sheffield and Ramin, 2004). However, one of the new approaches for accurate and rapid detection of microbial pathogens is a polymerase chain reaction (PCR) followed by fluorescence detection during the amplification (real time PCR) or after its termination [PCR– fluorescent amplification based specific hybridization (FLASH)] (Skottman et al., 2006; Wang et al., 2004; Bell et al., 2002).

The result analysis in these methods is carried out without the use of electrophoresis, which almost completely eliminates the cross over contamination with amplicons and therefore, eliminate the false-positive results. Nevertheless, it should be noted that the FLASH-PCR method requires no expensive equipments, as in the case of the real-time PCR. In the present study, we described a FLASH based PCR assay for detection of *C.*

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Table 1. Sequences of primers and probe used in this study.

Agent	Target gene	PCR product length	Primer and probe Seq.
C. tetani	TeT X	203 bp	F: 5´- CCGAAAGATGGAAATGCCT -3´ R:5´- GGATCGTTGCCTATTTGACC -3´ Probe: 5´-FAM- cccgtgggatcgttgcctatttgaccacacggg-BHQ1-3´

tetani, to be used in a diagnostic laboratory.

MATERIALS AND METHODS

Deoxyribonucleic acid (DNA) extraction

C. tetani (American Type Culture Collection (ATCC): 19406) was originally obtained from the ATCC, USA. In this study, *C. tetani* was grown in 5 ml LB-broth with shaking at 37°C for 16 h. Bacterial genomic DNA was isolated from pure cultures following procedures described earlier by Naqvi et al. (2003). The bacterial suspension was boiled for 20 min, and then centrifuged at 4000 rpm for 30 min. The supernatant was collected as PCR template and stored in 2 ml sterile tubes at -20°C until use.

Primer and probe design

Specific primers (Tetx F and Tetx R) and probe (Tetx TM) were designed based on the Tetx nucleotide sequence of the plasmid pE88 (NCBI accession No. AF528097). Primers and probe were designed using the Molecular beacon version 7.1 software (Biosoft Corporation, USA). All oligonucleotides were synthesized by Bioneer Company (Korea).

The fluorescent reporter dye at the 5′ end of the probe was 6-carboxyfluorescein (FAM), and a non-fluorescent quencher (BHQ1) was at the 3′ end. The sequences of primers and probes are shown in Table 1.

Detection by polymerase chain reaction (PCR)

All PCR assays were carried out in a DNA technology thermocycler (DNA Technology, Russia) in a total volume of 25 µl containing 12.5 µl 2XTaqMan Universal PCR master mix (Genet Bio, Korea) with the reaction buffer, dNTPs, $MgCl_2$, the 900 nM primers, 300 nM probe and 2.5 µl of the DNA template. Standard PCR thermocycling conditions were used: 10 min at 95°C, 40 cycles of 15 s. at 95°C and 1 min at 60°C.

Agarose gel electrophoresis and fluorescent amplification-based specific hybridization

PCR products were analyzed by electrophoresis in a 1.5% agarose gel in TAE (40 mMTris-HCl, 20 mM acetic acid, 1 mM EDTA) containing 0.5 µg/ml Ethidium bromide and the subsequent visualization using an ECX-15-M transilluminator (Vilber-Lourmat, France). Images were saved with the help of a chamber for documenting gels (GelImager 2, Russia), and also with the help of the fluorescence detector gene (ZAO NPF DNA technology) and the corresponding program that allows the visualization and storing the results of analysis. The obtained fluorescence data were analyzed using Gene software, version 4.4.1 (DNA Technology). A 100 bp DNA marker (DIALAT Ltd., Russia) was used.

RESULTS

C. tetani and other members of the clostridium group exhibit an extremely high degree of genomic homology (Peter et al., 2008). Thus, they make differentiation of these species challenging. In this study, we designed an assay for the detection of Tetx gene using the Flash fluorescent instrument after PCR reaction.

A gel electrophoresis analysis of the PCR for *C. tetani* (Figure 1) showed that, with the use of primers strictly specified for this pathogen, only the *C. tetani* is clearly identified (lane 4), whereas the amplification with the DNA of other Clostridium gives no amplicon.

The identification of *C. tetani* using the FLASH format (Figure 2) gave a clear unambiguous results similar to those obtained by the electrophoresis analysis in the determination of *C. tetani*.

DISCUSSION

Tetanus is a disease characterized by an acute onset of painful muscular contractions, generalized muscle spasms without other apparent medical causes and hypertonia (Edlich et al., 2003). Although, tetanus remains relatively rare in developed countries, it is important to be aware of its fatal condition, particularly in the older population. The fact that the patient did not manifest any symptoms of localized or generalized tetanus could be attributed to prompt management when a person presented to its primary care (Laverse et al., 2009). Recently, conventional PCR assays have been considered for many microorganisms. However, this assay has not been widely implemented in clinical laboratories, in part due to its requirement for time-consuming and labor-intensive steps, which include specimen preparation, amplification and product confirmation. Moreover, there have been concerns about amplification product (amplicon) carryover contamination (Herzog et al., 2009; Franke-Whittle et al., 2005; Peruski et al., 2002).

The principle of the PCR–FLASH method has been described in detail previously, in papers devoted to diagnostic test systems for Septoriatritici and Stangosporanodorum (Abramova et al., 2008), and some most widely occurring potato viruses (Ryazantsev et al., 2009; Ryazantsev et al., 2008). The FLASH-PCR assay described herein appears to be a useful test for detection

Figure 1. Electrophoresis in 1.5% agarose gel of product of the amplification of *C. tetani*. Lane 1, Negative control; Lane 2, 100 bp DNA marker;Lane 3, *C. acetobutili*; Lane 4, *C. tetani* 203 bp; Lane 5, Butolinum.

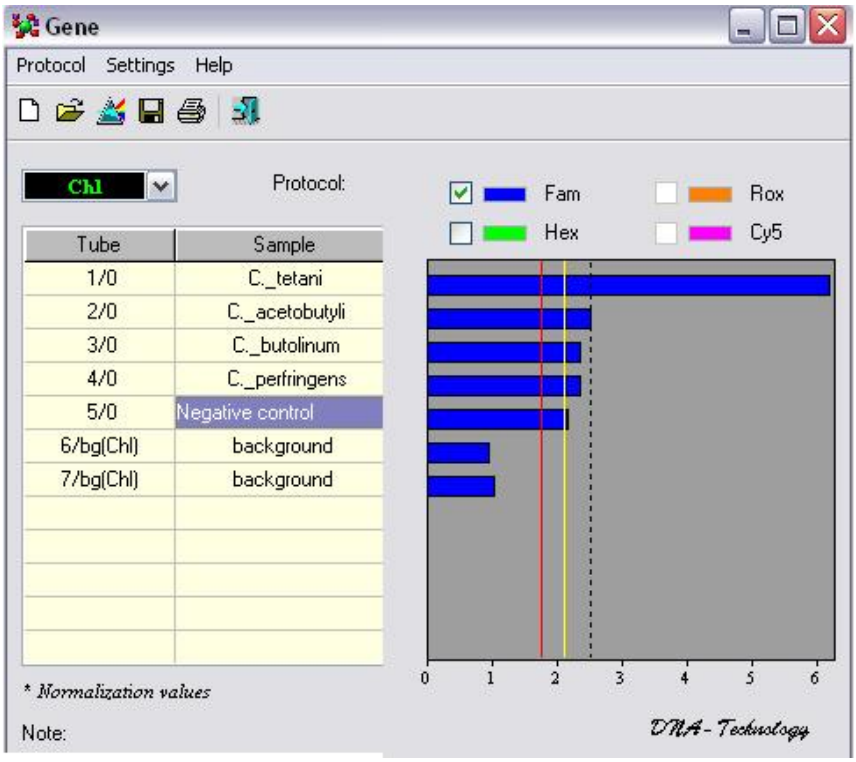

Figure 2. Diagnosis of *C. tetani* by FLASH-PCR method.

of *C. tetani*. The specificity of the assay was also demonstrated. None of the Clostridiums had positive results. These studies may be difficult in view of the very low incidence of human Tetanus infections.

Several previous studies have evaluated individual real-time PCR and microarray methodologies for the detection of *C. tetani* species (Peruski, 2002; Akbulut et al., 2005). However, the aim of our study was to develop a method which could be used for rapid detection and identification of *C. tetani*, in a mobile diagnostic laboratory setting. However, further clinical studies are required to determine the utility of this test for the rapid identification of *C. tetani* directly from human specimens.

In conclusion, the results of the present study indicated that the FLASH based PCR assay appear to be a suitable and rapid method for identification of cultured isolates of *C. tetani*. The method takes about 1 h to operate and it should be noted that the PCR–FLASH method requires no expensive equipment, as in the case of the real time PCR.

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REFERENCES

Abramova SL, Ryazantsev DYu, Voinova TM, Zavriev SK (2008). Diagnostics of phytopathogen fungi Septoriatritici and Stagonosporanodorum by fluorescent amplification-based specific hybridization (FLASH) PCR. Bioorg. Khim., 34(1): 107–113.

Akbulut D, Grant KA, McLauchlin J (2005). Improvement in laboratory diagnosis of wound botulism and tetanus among injecting illicit-drug users by use of real-time PCR assays for neurotoxin gene fragments. J. Clin. Microbiol., 43(9): 4342–4348.

Bell CA, Uhl JR, Hadfield TL, David JC, Meyer RF, Smith TF, Cockerill FR (2002). Detection of Bacillus anthracis DNA by light cycler PCR. Clin. Microbiol., 40(8): 2897–2902.

Brook I (2008). Current concepts in the management of Clostridium tetani infection. Expert Rev. Anti. Infect. Ther., 6(3): 327-336.

Edlich RF, Hill LG, Mahler CA, Cox MJ, Becker DG, Horowitz JH, Nichter LS, Martin ML, Lineweaver WC (2003). Management and prevention of tetanus. J. Long. Term. Eff. Med. Implants., 13(3): 139-154.

Franke-Whittle IH, Klammer SH, Insam H (2005). Design and application of an oligonucleotide microarray for the investigation of compost microbial communities. J. Microbiol. Methods 62(1): 37-56.

Herzog AB, McLennan SD, Haas CN, Rose JB, Pandey AK, Gerba CP, Hashsham SA (2009). Implications of Limits of Detection of Various Methods for Bacillus anthracis in Computing Risk to Human Health. App. Environ. Microbiol., 75(19): 6331–6339.

Laverse E, Dhamija K, Meyers M, Grant K (2009). Pre-emptive treatment for Clostridium tetani: importance of early recognition and treatment in the community. BMJ Case Reports, 11 Jun 2009, DOI 10.1136/bcr.03.2009.1649.

Naqvi A, Tinsley E, Khan SA (2003). Purification and characterization of the PcrA helicase of Bacillus anthracis. J. Bacteriol*.,* 185(22): 6633-6639.

Peruski AH, Johnson LH, Peruski LP (2002). Rapid and sensitive detection of biological warfare agents using time-resolved fluorescence assays. J. Immunol. Methods 263(1): 35–41.

Peter FH, Binnewies TT, David WU (2008). The genome BLAST atlas-a GeneWiz extension for visualization of whole-genome homology. Mol. Biosyst., 4(5): 363–371.

Poudel P, Budhathoki S, Manandhar S (2009). Tetanus. Kathmandu Univ. Med. J., (KUMJ), 7(3): 315-322.

Ryazantsev DYu, Abramova SL, Evstratova SV, Gagkaeva TYu, Zavriev SK (2008). FLASH-PCR diagnostics of toxigenic fungi of the genus *Fusarium*. Russ. J. Bioorg. Chem*.,* 34(6): 716-724.

Ryazantsev DYu, Zavriev SK (2009). An efficient diagnostic method for the identification of potato viral pathogens. Mol. Biol., 43(3): 515-523.

Sheffield JS, Ramin SM (2004). Tetanus in pregnancy. Am. J. Perinatol., 21(4): 173-182.

Skottman T, Piiparinen H, Hyytiäinen H, Myllys V, Skurnik M, Nikkari S (2006). Simultaneous real-time PCR detection of *Bacillus anthracis*, *Francisellatularensis* and *Yersinia pestis*. Eur. J. Clin. Microbiol. Infect. Dis., 26(3): 207–211.

Wang SH, Wen JK, Zhoua YF, Zhang ZH, Yang RU, Zhang JB (2004). Identification and characterization of Bacillus anthracis by multiplex PCR on DNA chip. Biosens. Bioelectron*.,* 20(4): 807–813.