Correspondence

Multiplex PCR for the detection of *Clostridium botulinum* & *C. perfringens* toxin genes

Sir,

Food-borne diseases constitute a major public health problem. Due to increased morbidity and mortality leading to time loss in the workplace and reduced productivity, food-borne diseases across the world cost billions of dollars annually. The *Clostridium* group of bacteria is commonly found in soil everywhere in the world, and some of its species live harmlessly in our intestines. *Clostridium* produces a chemical that is toxic to human body. One of the major illnesses caused by the *Clostridium* group of bacteria is food poisoning which includes *perfringens* food poisoning by *C. perfringens* and food-borne botulism by *C. botulinum*. The enterotoxin produced by *C. perfringens* and neurotoxin produced by *C. botulinum* are responsible for the illness.

One of the inherent difficulties in the detection of food pathogens is that they are generally present in very low numbers (<100 cfu/g) in the midst of up to a million or more other bacteria and these microbes may be lost among a background of indigenous microflora in the samples. The isolation of these organisms in the samples is frequently complicated by the presence of nontoxigenic strains that phenotypically and genetically resemble and exhibit a high relatedness with their toxigenic counterparts. Diagnosis of these food pathogens is confirmed by clinical observation together with the detection of toxin in a patient or suspected food sample. Presently, the only method of sufficient sensitivity for detection of botulinum neurotoxin is the mouse lethality test or bioassay, which is not only time-consuming and expensive but also raises ethical concerns.

For *C. botulinum* and *C. perfringens* several PCR-based detection methods have been reported during the last decade. Compared to conventional methods, these protocols provide rapid and sensitive detection of these organisms. However, none of the reported PCR was able to detect the enterotoxin gene and neurotoxin gene simultaneously. The multiplex PCR method may provide a more sophisticated approach, enabling a simultaneous and specific detection of *C. perfringens* and more than one serotype of *C. botulinum*.

We therefore attempted to standardize a multiplex PCR using a pair of *C. perfringens* enterotoxin primer (melting temperature, 55°C) and two pairs of *C. botulinum* neurotoxin-specific primers with almost equal melting temperatures (60°C) for the simultaneous detection of *C. perfringens* and *C. botulinum* toxin genes using reference strain DNA in the microbiology department of All India Institute of Medical Sciences (AIIMS), New Delhi. The sensitivity and specificity of the assay was also analyzed. A few suspected cases of food poisoning were analyzed to see the applicability of this assay.

The DNA of enterotoxigenic strain *C. perfringens* type A - CN 3418 cpe +, *C. botulinum* type A (proteolytic *C. botulinum* types A, ATCC 25763) and B (nonproteolytic types B, Eklund 2B) were used for the standardization of multiplex PCR. Other *Clostridium* species including *C. sporogenes*, *C. bifermentans*, *C. hastiforme* as well as other bacteria *viz.*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, and *Pseudomonas aeruginosa* were used to confirm the specificity of PCR.
A total of 8 suspected cases of botulinum food poisoning (age group of 4 to 20 yr) received in the Anaerobic Laboratory, Microbiology Department, All India Institute of Medical Sciences, during the period of 1 year (January 2005 - December 2005) were analyzed using this assay. Total genomic DNA of _C.perfringens_ reference strain were extracted by the boiling method. All the diarrhoeal stool samples (0.1 g) were enriched overnight in Robertson's Cooked Meat broth (RCM), and DNA was extracted by Instagene Matrix (BioRad, USA).

The primers for the enterotoxin gene (cpe) of _C.perfringens_ was chosen from the sequences that had been developed and validated by Fach and Popoff to yield a 426 bp fragment for the _cpe_ gene and for _C. botulinum_, the primers were selected from the nonhomologous regions of the BoNT types A, and B gene.

The PCR analysis was carried out in 25 µl volume. The mixture contained 10 mM Tris HCl, 50 mM KCl, 0.0001 per cent gelatin, 3 mM MgCl₂, 100 µM of dNTPs (MBI, Fermentas, USA), 50 pmols of primers of _C. botulinum_ A and B and 20 pmols of primers of _C.perfringens_ enterotoxin (BioBasic Inc, Canada), 1.5 U of Taq polymerase (MBI, Fermentas, USA) and 1 µl of DNA. PCR analysis was performed by initial denaturation step at 95°C for 30 sec, followed by 25 cycles of amplification (denaturation at 95°C for 30 sec, primer annealing at 60°C for 25 sec, primer extension at 72°C for 1 min 25 sec) and 15 cycles of amplification (denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, primer extension at 72°C for 30 sec) ending with a final extension at 72°C for 10 min using a thermocycler (Gene Amp PCR system 9700; AB Applied Biosystems, USA).

Specificity of the PCR assay was evaluated using several isolates belonging to the genus _Clostridium_ and other isolates belonging to other genera. Sensitivity of PCR was confirmed using DNA of the reference strains. A 10-fold dilution of purified DNA was used from type A and type B _C.botulinum_ and enterotoxigenic _C.perfringens_ prepared in distilled water. The highest dilution yielding an amplicon with 3 bands (426 bp of _C.perfringens_ and 782 and 205 bp of _C.botulinum_) in multiplex PCR from a 10 µl sample volume was taken as the end point.

_C. botulinum_ types A, B and enterotoxigenic _C.perfringens_ yielded the expected amplification products: type A, 782 bp; type B, 205 bp; enterotoxigenic _C.perfringens_ type A 426 bp. The mixed DNA suspensions yielded the corresponding DNA fragments (Fig.). The PCR products were clearly visualized in agarose gels; a 150 to 200 bp difference in the size of each amplification product enabled an easy distinction between the fragments without the use of high-resolution agarose. No detectable amplification occurred with DNA from other bacterial species analyzed. The sensitivity of multiplex PCR was 20-25 pg/10 µl respectively. All expected amplification products were easily differentiated in low-resolution agarose gels.

![Fig. Multiplex PCR for the detection of _C. perfringens_ enterotoxin and _C. botulinum_ neurotoxin gene. Lanes: 1, molecular weight marker; 2,4, enterotoxigenic _C.perfringens_ type A; 3, _C. botulinum_ type B; 5, _C. botulinum_ type A and enterotoxigenic _C.perfringens_ type A; 6 & 7, _C. botulinum_ types A, B, and enterotoxigenic _C.perfringens_ type A; 8, negative control.](image-url)
Of the eight diarrhoeal cases analyzed, none showed the presence of neurotoxin or enterotoxin genes. All these samples were further analyzed for the presence of C. perfringens phospholipase C gene by PCR described previously and enterotoxin by reverse passive latex agglutination (RPLA) and ELISA. Two of the analyzed samples were positive for phospholipase C gene (283 bp) which shows the presence of C. perfringens in the samples. The possibility of C. perfringens as the causative agent of food poisoning in these cases was ruled out as all of them were negative for enterotoxin by PCR, RPLA and ELISA.

This multiplex PCR might be useful in the diagnosis of food-borne clostridial pathogens—C. botulinum and C. perfringens, since the previously described methods require more than one PCR for simultaneous detection and identification of C. botulinum types and enterotoxigenic C. perfringens. The assay can thus markedly improve the PCR diagnostics of food-borne clostridial infections. Further, this may be used to overcome the cumbersome and time consuming animal tests used to identify toxigenic Clostridium. Its application for the direct detection of pathogenic Clostridium in food samples needs to be evaluated further.

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