



Original Article

Investigation of Botulinum Neurotoxin Types from *Clostridium botulinum* Causing A Recent Outbreak in Vietnam

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Abstract: *Clostridium botulinum* is one of the causes of undiagnosed sudden deaths in human due to the lethal botulinum neurotoxins (BoNTs). Foodborne botulism rarely occurs in developed countries because of being closely monitored, in opposite to developing countries including Vietnam. In the August 2020 food poisoning outbreak in Vietnam, the presence of *Clostridium botulinum* and BoNTs was identified by culture and mouse bioassay, however, information regarding the possible toxin types was unclear. To examine the types of toxin, we designed primers for specific amplification of gene regions encoding the light chain (LC) domains for both BoNT/A and BoNT/B. After optimization, the expected PCR products were sequenced and analysed. The results showed that the sequence of gene encoding BoNT/A LC was 99.8% identical to the CB-27 strain. The sequence of gene encoding BoNT/B LC was approximately 98.8% identical to reference strains. Additionally, we analyzed the deduced amino acid sequences of the inferred proteins and identified a substitution that resulted in a frameshift and premature stop codon as previously found in a defective form of BoNT/B. Collectively, we provided the first evidence for *C. botulinum* strain possessing A(b) type in this studied outbreak. Further enzyme activity and neutralization assays are necessary to validate this preliminary toxin typing.

Keywords: *Clostridium botulinum*, BoNT neurotoxin, *bont* genes, light chain.

1. Introduction

Clostridium botulinum is a Gram positive, rod shaped, spore forming, anaerobic that produces botulinum neurotoxins (BoNTs). Botulinum is the cause of poisoning in humans

and animals with different symptoms including vomiting, nausea, muscle weakness, blurry vision, difficult breathing or even death [1]. Botulism progresses rapidly, leading to death if not diagnosed and treated properly. Recovery from the symptoms of botulism is slow. To date, botulism has been reported in many countries. In the United States, there are 70-100 cases of botulism each year [2]. From August,

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2020 to now, Vietnam has recorded many botulinum poisoning cases due to the consumption of vegan pate (more than 40 cases) or other types of food. Botulinum antitoxin is not available, expensive and imported from abroad. Botulism antitoxin only works for the first week after poisoning, so identify the cause of poisoning is essential [1].

BoNTs are classified into eight types (A-H), corresponding to eight types of serum. Types A, B, E, F are harmful to humans [3]. BoNT/A is more potent than BoNT/B, BoNT/E with persistent symptoms. In addition, the treatment process for botulism with varied symptoms is different, thus early determination of toxin type guides the proper and effective treatment. BoNTs are polypeptide chains with approximately 1300 amino acids in length. BoNTs have three subunits including the light chain LC (50 kDa) with catalytic function, the heavy chain HC (100 kDa) consisting of the translocation domain and the receptor binding domain. The LC has endopeptidase activity, cleaving SNARE proteins preventing their assembly and inhibiting the release of acetylcholine [4]. The study by Pellet et al., demonstrated that the LC plays more roles than the HC in the toxins. Particularly, LC domain induced virulence, defined the duration of toxin action in mouse and cleaved SNAP-25 protein in cultured cells [5].

In nature, *bont* gene exists in *bont* gene clusters or genes coding BoNTs- associated proteins, that transport the toxins across plasma membranes or help spores survive in adverse conditions [6]. *bont* genes encoding toxins A-H were different by at least 2.6% in amino acid sequence [7]. A *C. botulinum* strain is capable of carrying several genes encoding multiple types of toxin due to recombination or horizontal transfer between homologous genes. For instance, a study showed that recombination occurring in the *ntnh* gene cluster caused the integration of *bontA1* genes into the *bontB* gene cluster [8]. As a result, a *C. botulinum* strain may express different toxins equally or a strong-expressed type and a poorly-expressed type.

In this study, we determined DNA sequences, then analyzed the deduced protein sequences of BoNT/A-LC and BoNT/B-LC of *Clostridium botulinum* strains isolated in a recent outbreak in Vietnam. The result provided the first evidence for the presence of *C. botulinum* strains possessing A(b) type (dominant BoNT/A and silent BoNT/B).

2. Materials and Methods

All strains used in this study were isolated from food and stool samples and provided by National Institute for Food Control (NIFC), including NIFC 671, NIFC 672, NIFC 673, NIFC 674, NIFC Cb. NIFC Cb was confirmed to carry BoNT/B. Genomic DNA was extracted and was quantified using a spectrophotometer (NanoDrop, US).

The light chain of *bontA* and *bontB* genes was amplified from the genomic DNA of *Clostridium* strains by PCR using the following primer: *bontA* (forward: 5'-ATTATAAGAGGIGTAAATATGC-3', reverse: 5'-AAGGACTAAAAACAAGTCC-3'); *bontB* (forward: 5'-

AAAATAAGGAGGAGAATATTTATGCC-3', reverse: 5'-CTGGAGCTTTAACACTTTTACAC-3'). Dream Taq DNA Polymerase (Thermo Fisher Scientific, US) in a reaction containing 1X Dream Taq buffer, 2 mM MgCl₂; 0.2 mM dNTP mixture; 10 pmol forward primer and 10 pmol reverse primer and 20 ng of the genomic DNA. The PCR conditions were as follows: 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 60s, annealing at 50 °C for 60 s (*bontA*) or 53 °C for 60s (*bontB*), extension at 72 °C for 90s and 72 °C for 5 min. The products of the reaction were observed by gel electrophoresis on 2% agarose under ultraviolet light, which was then sequenced. The sequences were analyzed using the BioEdit and Mega X software and compared other sequences in the NCBI database nucleotide. Phylogenetic trees were constructed using Maximum Likelihood (bootstrap replications are 1000 times).

3. Results

3.1. Optimization of PCR Conditions for BoNT/A-LC and BoNT/B-LC Amplification

Based on the annealing temperatures of primers and the lengths of target domains, we set up the initial PCR conditions. PCR amplification for *bontA* gene was performed at 50 °C for 90 s for annealing stage, resulting in a single faint band. However, when the annealing time was reduced to 60 s, we observed a bright band of approximately 1.4 kb (Figure 1, lanes labeled NIFC 671, 672), the expected size according to theoretical calculation. For *bontB*, the initial T_m (50 °C) was tried for 60 s and 90 s, producing a fuzzy band of 250 bp, incorrect size for the target fragment. To enhance the specificity, the T_m was increased to 53 °C for 60 s, resulting in specific PCR products of approximately 1.3 kb in size, as shown in Figure 2, lanes labeled NIFC 671, 672, Cb.

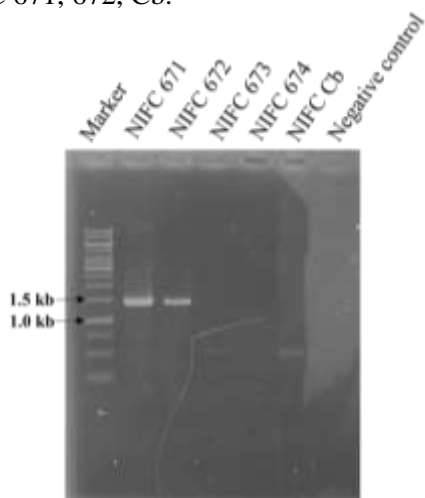


Figure 1. PCR products of *bontA* gene.

According to the PCR results, *bontA* gene was identified in 2 out of 5 samples including NIFC 671 and NIFC 672, while *bontB* gene was present in 3/5 samples. Strains NIFC 671 and NIFC 672 contained the gene regions encoding BoNT/A-LC and BoNT/B-LC. These conclusions were consistent with the results using standard method of TCVN 11395:2016 that determined toxin types (A, B, E, F)

by multiplex PCR targeting shorter fragments (100-550 bp) in LC domains.

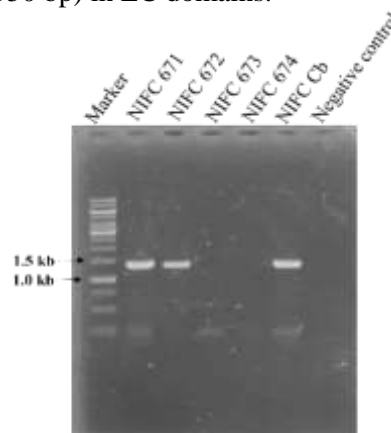


Figure 2. PCR products of *bontB* gene.

The distribution and prevalence of toxin types depend on geographic locations. BoNT/A and BoNT/B are commonly found in warm temperate regions [9]. Foodborne BoNT/E was detected in the coastal of Northern Europe (Baltic) and Alaska, which was associated with aquatic animals [10]. In Asia (Thailand and China), *C. botulinum* types A, B are common with the higher frequencies than others [11, 12]. Strains NIFC 671 and 672 isolated in Vietnam carried genes encoding BoNT/A-LC and BoNT/B-LC, preliminarily demonstrating the similarity of toxin types in the same regions.

3.2. Phylogenetic Analyses

We constructed phylogenetic tree based on sequences of *bontA* gene and *bontB* gene. Phylogenetic analysis showed that strains NIFC 671 and 672 most closely related to strains NCTC 13319 and CB-27 (Figure 3). Two reference strains were identified as type A, strain CB-27 expressed only BoNT/A despite carrying both genes [13].

Phylogenetic tree based on *bontA* showed that strain NIFC 672 closely related to the branch carrying strain NIFC 671 with a bootstrap support of 64%, generally considered not strong (Figure 3). The sequence of gene encoding BoNT/A-LC of both strains was approximately 99.8% identical to the CB-27 strain (Genbank Accession No. MT199282).

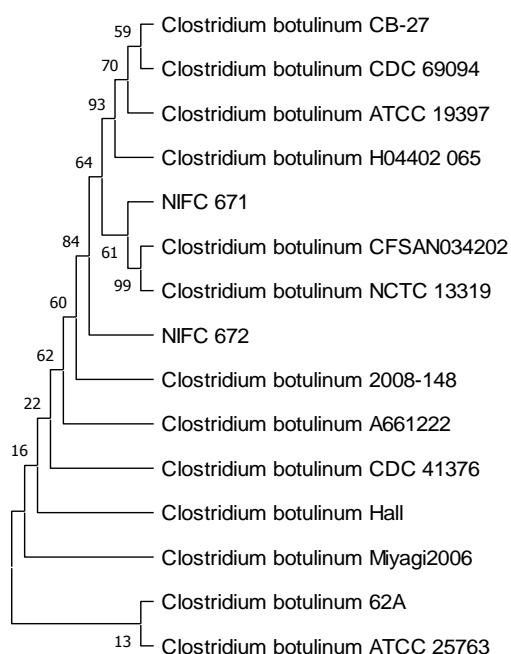


Figure 3. Phylogenetic tree of two Vietnamese *C. botulinum* strains based on the LC sequences of *bontA*.

The two strains split into a separate branch with reference strains. The branch, including NIFC 671 and NIFC 672, had higher identity compared to strain 2008-148 with a bootstrap support of 84% [14]. Therefore, the NIFC 671 and 672 strains isolated in Vietnam could be different from the published strains.

For *bontB* gene, the data produced lower bootstrap support for NIFC 671 and NIFC 672 (41% and 64%, respectively). The *bontB* sequences in strain NIFC 671 shared 98.6% identity to the sequence in strain NCTC 3807 of subtype B1 [15]. The *bontB* sequence of strain NIFC 672 was 98.7% identical to strain Saraburi 2010 of type A1(B) [11].

The phylogenetic tree based on LC sequences of *bontB* and showed the differences of *C. botulinum* strains isolated in Vietnam from other strains, which were characterized in the Asian countries (Thailand, Korea, Japan) and other regions (United States, Germany, Canada). In this paper, the LC sequences used for phylogenetic trees construction were about 1000 nucleotides long, approximately 25% of the

full genes encoding toxins (4000 nucleotides) [16]. Therefore, we proposed that the analysis of phylogenetic trees among strains could be improved by sequencing the remaining domains.

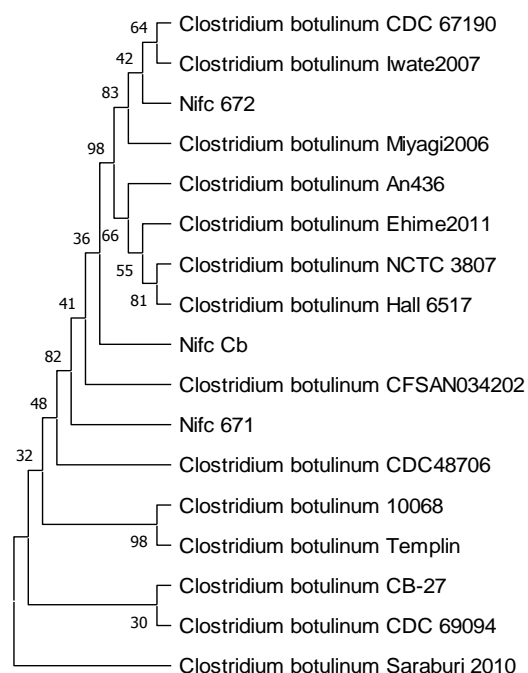


Figure 4. Phylogenetic tree of two Vietnamese *C. botulinum* strains based on the LC sequences of *bontB*.

3.3. Protein Sequences Analysis

Sequences alignments of BoNT/A-LC from strains NIFC 671 and NIFC 672 showed high homology (99%) with strain CB-27. Some non-homologous amino acids located at the two ends of polypeptide chain (Figure 5.).

Analysis of BoNT/B-LC indicated several substitutions, resulting from different amino acid codons. Here, a 328G → T mutation changed the amino acid at position 128 from Glu → Stop (GAG to TAG), causing a truncated protein. The active site region of LC resided in residues 268-400, so changes in amino acids can be very important to the protein function. Thus, we predicted that BoNT/B-LC was inactivated as previously found in a defective form. The same substitute in mutation occurred in the study of Roger [17].

Strain NIFC Cb did not show mutations in protein sequence (Figure 5).

Nifc 671	RRVPL	-	EFNTNIAS
Nifc 672	RRVPL	-	EFNTNIAS
Nifc Cb	RRVPL	EE	EFNTNIAS
<i>C. botulinum</i> NCTC 3807	RRVPL	EE	EFNTNIAS

Figure 5. Comparison of amino acids of BoNT/B-LC.

For BoNT/A-LC and BoNT/B-LC of protein sequences, we proposed that strains NIFC 671 and 672 could produce BoNT/A only, not BoNT/B-LC. NIFC Cb carried *bontb* gene.

4. Conclusion

Overall, we sequenced and analyzed genes encoding BoNT/A-LC and BoNT/B-LC of *C. botulinum* strains isolated in a recent outbreak in Vietnam. The results showed the strains NIFC 671 and 672 possess the gene encoding botulinum toxin type A. There observations initially identified the type of toxin causing botulism, however, further investigation of the activity and toxicity is required to confirm the toxin types.

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