การระบาดของ Type B Botulism ที่จังหวัดชัยภูมิ ประเทศไทยในปี 2014

An Outbreak of type B botulism in Chaiyaphum Province, Thailand 2014

ปิยะดา หวังรุ่งทรัพย์* ชุติมา จิตตประสาทศีล กรัณย์ สุทธิวราคม
Piyada Wangroongsarb* Chutima Jittaprasartsin Karun Suthivarakom
ธนิตชัย คำแถลง นัฐพงษ์ ชืนบาน สมชาย แสงกิจพร
Thanitchai Kamthalang Nattapong Cheunban Somchai Sangkitporn

สถาบันวิจัยวิทยาศาสตร์สาธารณสุข กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข จ.นนทบุรี
National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi Province

*ผู้รับผิดชอบบทความ (Email : piyada.w@dmsc.mail.go.th)

Received December 2014
Accepted as revised January 2015

Abstract

Introduction: *Clostridium. botulinum* strains that produce types A and B to in generally associated with several outbreaks in the United States, China, South Amerca and southern European countries, and the most frequently implicated foods are vegetables. Diagnosis of botulism has been made by detecting the neurotoxin and *C. botulinum* cells in patients and/or suspected food samples. The aim of this study was to identify the cause of foodborne outbreak and analysis the symptoms of botulism in Chaiyaphum Province, 2014.

Materials and methods: The samples from Chaiyaphum Province outbreak were comprised of suspected food and clinical samples from 4 patients. Total 11 samples were identified by the cultivation, mouse bioassay and typing toxin genes amplication by multiplex PCR. The subtypes B1-B8 were compared based on the amino acid sequences alignment of bont/B1 to B8 subtypes using MEGA software which produce an unweighed pair group method with arithmetic mean (UPGMA).

Results: The clinical symtoms were observed especially with blurred vision, glossoplegia, dysarthria, nausea, dyspnea and required mechanical ventilation for support breathing. The etiological agent of this foodborne botulism outbreak detected by culturing, mouse bioassay and multiplex PCR method in fermented bamboo shoots contaminated was *C. botulinum* type B8. The phylogenetic tree of Chaiyaphum 2014 strain was constructed and the result demonstrated an identical to B8 subtype of Surat Thani 2012 strain (KC 714045).

Conclusions: From this study showed that the etiologic agent of an outbreak in Chaiyaphum Province was fermented bamboo shoot contaminated with *C. botulinum* type B8. Botulism disease must be considered clinical symptoms which was important to provide treatment of patients in time. It should combine with laboratory diagnosis in order to obtain the accurate results.

Bull Chiang Mai Assoc Med Sci 2015; 48(1): 49-58. Doi: 10.14456/jams.2015.3

Keywords: *C. botulinum*, foodborne, molecular technique

บทคัดย่อ

บทนำ: *Clostridium botulinum* ที่เป็นสายพันธุ์ที่ผลิตสารพิษชนิด A และ B เกี่ยวข้องกับการแพร่ระบาดในประเทศสหรัฐอเมริกา-จีน-อเมริกาใต้และประเทศในยุโรปตอนใต้และอาหารส่วนใหญ่ที่เกี่ยวข้องเป็นผัก การตรวจวินิจฉัยโรคโดยการตรวจสอบ neurotoxin และการเพาะเลี้ยงเชื้อ *C. botulinum* ในผู้ป่วยและ/หรือตัวอย่างอาหารที่สงสัยจุดประสงค์ของการศึกษาครั้งนี้เพื่อหาสาเหตุของการระบาดและวิเคราะห์อาการของโรค Botulism ที่เกิดในจังหวัดชัยภูมิ ปี 2014

วัสดุและวิธีการวิจัย: ตัวอย่างจากการระบาดที่จังหวัดชัยภูมิประกอบด้วยตัวอย่างอาหารที่สงสัยและสิ่งตัวอย่างจากผู้ป่วย 4 คน รวมทั้งหมด 11 ตัวอย่างนำมาทำการทดสอบด้วยวิธีการเพาะเลี้ยงเชื้อ การทดสอบหาสารพิษในหนูทดลองและวิธีทางชีวโมเลกุล เชื้อ *C. botulinum* ทำ subtyping โดยการเปรียบเทียบการจัดตำแหน่งของกรดอะมิโนของ *bont/B1-B8* และใช้ software MEGA คำนวณแบบ unweighed pair group (UPGMA)

ผลการศึกษา: อาการทางคลินิกที่สังเกตเห็นชัดโดยเฉพาะคือ ตาพร่ามัว ลิ้นแข็ง ความผิดปกติเกี่ยวกับการพูด คลื่นไส้และการหายใจขัดซึ่งต้องอาศัยเครื่องช่วยหายใจ ส่วนสาเหตุของการระบาดครั้งนี้คือ หน่อไม้ปี๊บที่มีการปนเปื้อนเชื้อ *C.botulinum* type B8 ซึ่งได้ผลจากการเพาะเลี้ยงเชื้อ การทดสอบหาสารพิษในหนูทดลองและวิธีทางชีวโมเลกุล ผลจากการสร้าง Phylogenetic tree โดย subtyping B1-B8 ของเชื้อ *C. botulinum* สายพันธุ์ชัยภูมิ 2014 พบว่าเป็น subtype B8 ซึ่งเหมือนกันกับสายพันธุ์สุราษฎร์ธานี 2012 (KC 714045)

สรุปผลการศึกษา: จากการศึกษาครั้งนี้พบว่าสาเหตุของการระบาดที่จังหวัดชัยภูมิคือหน่อไม้ปี๊บที่มีการปนเปื้อนเชื้อ *C.botulinum* type B8 การพิจารณาอาการทางคลินิกมีความสำคัญเพื่อให้การรักษาผู้ป่วยได้ทันเหตุการณ์ร่วมกับการตรวจวินิจฉัยทางห้องปฏิบัติการเพื่อให้ได้ผลวินิจฉัยโรคที่ถูกต้อง

วารสารเทคนิคการแพทย์เชียงใหม่ 2558; 48(1): 49-58. Doi: 10.14456/jams.2015.3

คำหัส: *C.botulinum*, foodborne, molecular technique

Introduction

Clostridium botulinum produces a characteristic botulinum neurotoxin (BoNT), which is classified by Centers for Disease Control and Prevention as one of the six highest risk threat agents for bioterrorism (Category A agent).[1] Botulinum neurotoxin (BoNT) exists into 8 toxin types (A-H), distinguished by the neutralization of toxin using type-specific polyclonal antibodies and molecular bioloay methods.[2,3] Botulinum toxin types A, B, E, F, and H cause disease in humans. Types A, B, and E are associated with foodborne illness, especially type E associated with fish products. Type C produces limberneck in birds and type D causes botulism in other mammals. There is no disease associated with type G. An eighth toxin, type H, was discovered by researchers at the California Department of Public Health in 2013. Four of these (types A, B, E and rarely F) cause human bot lism. Types C, D and E cause illness in other mammals, birds and fish. *C. botulinum* strains that produce types A and B toxin were generally associated with several outbreaks in temperate and warmer regions. Proteolytic type toxins A and B are linked to majority of the outbreaks in the United States, China, South America and southern European countries, and the most frequently implicated foods are vegetables. In France 2014, an outbreak of human botulism was due to consumption of ham containing botulinum neurotoxins B and E.[4] In China 2014, two patients from Sichuan Province with symptoms of food-borne botulism, a rare but fatal

illness caused by the consumption of foods containing *C. botulinum* type A.[5] Also, occurring in Ontario, Canada 2014, two adult females consumed a meal consisting of a traditionally prepared salted fish contaminated with *C. botulinum* type E.[6] Laboratory investigation of botulism has been made by detecting of the neurotoxin and microorganism in patients and/ or suspected food samples.[7,8] Mouse bioassay is the only universal method for the detection of toxin-producing *C. botulinum*. It is highly specific and sensitive whereas it is time-consuming and involves in rigors animal ethical issues. Phylogenetic analysis and typing of *C. botulinum* by molecular biology methods such as pulsed-field gel electrophoresis (PFGE)[9] ribotyping (rRNA analysis), amplified fragment length polymorphism (AFLP),[10] multilocus sequence typing (MLST),[11] randomly amplified polymorphic DNA analysis (RAPD) and repetitive element sequence based PCR (Rep-PCR)[12,13] are currently in use. Detection of virulence factor(s) is a prerequisite for the identification of toxigenic *C. botulinum* strains and other related species for causing the botulism. The aim of this study was to identify the cause of foodborne outbreak and analysis the symptoms of botulism in Chaiyaphum Province, 2014.

Materials and methods

Observation of patients

Clinical observations recorded by nurse as a part of clinical information on admission to hospital. The symptom of observations must reflect the requirements of therapies. In certain clinical circumstances further observations (for example neurological symptoms) may be required.

Culture and isolation of *C. botulinum*

The outbreak in Chaiyaphum province comprised of 3 stool samples, 6 sera samples and 2 fermented bamboo shoots samples received during 22April to 1 May 2014. Stool and sera samples of the 1st-4th patients were received as showed in table 1. The fermented bamboo shoots were collected from the leftover (13-57-06653) and the same lot of the leftover collected from factory (13-57-06730). Epidimiology staffs collected all samples and sent for identification at the Anaerobic bacteria section, National Institute of Health, Thailand. One gram of food or stool specimens was weighed in a steriled container. The specimen was transferred to a steriled mortar and 1 mL of cold gelatin diluent buffer was added. The samples were centrifuged in refrigerated centrifuge at 12,000 x g for 20 minutes, and the supernatant were applied for toxigenicity test using mouse bioassay.[14] Half of pellet from samples was inoculated and grown on Egg Yolk Agar (EY) plates and incubated under anaerobic conditions at 35°C for 2 days. The results showed lipase positive which were suspected *C.botulinum*. The remaining of the pellet was divided into three parts and each was inoculated onto Chopped Meat-Glucose-Starch (CMGS) medium, CMGS medium heated at 80°C for 15 min, and Tryptone peptone glucose yeast extract trypsin (TPGYT). All of them were incubated in anaerobic conditions at 35°C for 5 days. All clinical isolates were characterized as followed by Dowell and Hawkins,[15] and biochemical testing by Holdeman.[16] The biochemical testing were SIM Test (hydrogen sulfide, indole, motile), hippurate hydrolysis Test, nitrate reduction test, gelatin, milk, esculin, the ferment of the peptone yeast extract containing starch, arabinose, dextrose, fructose, lactose, maltose, mannose, mannitol, salicin, sucrose, xylose. The results of suspected *C.botulinum* were milk digestion, gelatin and esculin hydrolysis positive. All suspected *C.botulinum* should confirm by mouse bioassay and molecular technique. Institute of Health Animal Care and Use Committee (NIH-ACUC) permit No. 57-017.

Mouse bioassay (MBA)

The mouse bioassay (MBA), a lethal assay, is the standard procedure for detection of BoNT. Mice are injected intraperitoneally with a sample suspected of containing toxin and monitored for as long as 4 day. BoNT potency is defined by the MBA and, by definition, the limit of detection is 1 LD_{50}. This value represents the results of serial dilutions of BoNT in which each dilution is injected intraperitoneally into 8-10 mice; 1 LD50 is the amount of toxin that produces botulism in 50% of

the mice at a single dilution (for example, if 10 mice were injected with 1 LD_{50}, only 5 would be expected to show signs of botulism). Although the MBA typically is positive within 24 h, many clinical specimens have low levels of toxin (less than 4 LD_{50}/mL) which may not be evident within 4 days. If a sample is positive for botulinum neurotoxin, mice develop ruffled fur, a narrowed waist (due to respiratory failure), and muscle weakness. The toxin type can be determined by adding type-specific antitoxins to aliquots of the diluted sample; mice injected with a toxin-containing sample that also contains antitoxin do not exhibit symptoms of botulism. The MBA has been used to detect BoNT in a wide variety of matrices, including serum, stool, vomitus, gastric content, enema wash with steriled water, food, tissue samples, and culture supernatants. Detection and typing of neurotoxin in serum and supernatant of culture were previously described by Hatheway et al.[14] The neutralization test was conducted using monovalent botulism antitoxin reagent and performed according by qualified laboratories from the Biological Reagents Program, CDC. The protocol was approved by the National

Identification of *C. botulinum* DNA by multiplex PCR

C. botulinum were grown in Wiklins Charlgen broth (WB), incubated anaerobically at 35°C for 48 h, and bacterial cells was harvested by low speed centrifugation. Cell pellets was re-suspended in 1 mL of distilled water and chromosomal DNA were extracted using the QIAmp DNA Mini Kit (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions and stored at 4°C until use. The multiplex PCR ultilized for BoNT identification was performed as previously described by Lindstrom et al.[17] The primers were as following, CBMLA1: 5'AGC TAC GGA GGC AGC TAT GTT3', CBMLA2: 5'CGT ATT TGG AAA GCT GAA AAG G3' for bont/A, CBMLB1: 5'CAG GAG AAG TGG AGC GAA AA3', CBMLB2: 5'CTT GCG CCT TTG TTT TCT TG3' for bont/B, CBMLE1: 5'CCA AGA TTT TCA TCC GCC TA3', CBMLE2: 5'GCT ATT GAT CCA AAA CGG TGA3' for bont/E and CBMLF1: 5'CGG CTT CAT TAG AGA ACG GA3', CBMLF2: 5'TAA CTC CCC TAG CCC CGT AT3' for bont/F. Thirty microliters of reaction composed of 0.5 µL of extracted DNA template, 0.1 µM of each primer, 200 nM of each deoxynucleotide triphosphate (dNTP), 5X Buffer, 1U of GoTaq® DNA polymerase and 1.5 mM $MgCl_2$ (Promega). PCR was done in 35 cycles and the profiles were as followed denaturation at 95°C for 20 sec, annealing at 55°C for 30 sec, extension at 62°C for 2 min. The amplified PCR products were electrophoresed in 2% agarose gels, and stained with ethidium bromide and visualized by UV transilluminator (Syngene, UK). All positive results were confirmed by mouse bioassay.

Sequence analysis of bont genes

The neurotoxin genes of Chaiyaphum 2014 strain were amplified by PCR using the available sequences on GenBank database. PCR was performed in a 50 µL reaction containing 1 ng extracted DNA, 0.5 µM of each primer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 5X Buffer and 2.5 U of GoTaq® DNA polymerase and 1.5 mM $MgCl_2$ (Promega). Each PCR cycle composed of denaturation at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and was repeated for 30 times. Final extension was carried out 72°C for 10 min. Amplicons were directly sequenced by primer walking and using the ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Phylogenetic analysis

DNA alignments were created with Clustal-W version 2.0 (http://www.clustal.org).[18] A phylogenic tree was constructed based on the sequences of bont/B using the unweighed pair group (UPGMA) with 1,000 bootstrap replication, and analysed by MEGA program version 5.0.[19]

Results

Observation of patients

During the Songkran day (April 15, 2014), all relatives would gather and ate together. The outbreak occurred in Chaiyaphum Province, the family gathering was 4 persons with 3 female and 1 male, aged between 21 and 38 years. After consumption of the fermented bamboo shoots, all of them had typical food poisoning

symptoms. Two persons stayed in the Chaiyaphum Province but one couple returned to Chonburi Province. On April 16, the first female patient with the aged of 27 years (Patient 1), had symptoms with severe pain in the lower right abdomen and admitted at Chaiyaphum Hospital. The doctor diagnosed suspected appendicitis. Upon slight recovery the patient was discharged on April 18. On April 19, she was admitted in hospital again with the symptom of blurred vision, dysarthria, dysphagia. On April 16, another 21-year-old female patient (Patient 2) with symptoms of nausea, vomiting and had diarrhea four times a day admitted in the Chaiyaphum Hospital. Two day later, she had symptoms of blurred vision, slurred speech, numbness of hands, dysphagia and drooping eyelids both. Both patients were diagnosed to be botulism and treated with antitoxin. The patients recovered and could spontaneously breathing after treatment. Likewise, the couple of patients consisted of a aged 38-year-old male (Patient 3) and a 33-year-old female (Patient 4) had symptom of headache, nausea on April 16, 2104, and one day later, they were admitted to Phyathai-Sriracha Hospital with similar symptoms and subsequently ventilated to support breathing and tansferred to the intensive care unit. On April 17, they were diagnosed to be botulism and were treated with antitoxin and the respiratory support indicated the severity of the illness. Eventually, they recovered under medical observation.

Identification of C. botulinum and detection of neurotoxin production

C. botulinum type B was identified in three stool specimens and one fermented bamboo shoots by culturing, and multiplex PCR whereas the remain sample was negative (Table 1). All supernatant samples were tested by mouse bioassay, the result of supernatant of all stools and fermented bamboo shoot (13-57-06653) were positive except the sera and fermented bamboo shoot (13-57-06730) as showed in table 1. The amplified PCR products for bont/A, B, E and F were 782 bps, 205 bps, 389 bps and 543 bps, respectively. The bont/B amplicon from Chaiyaphum 2014 strain was detected as shown in figure 1.

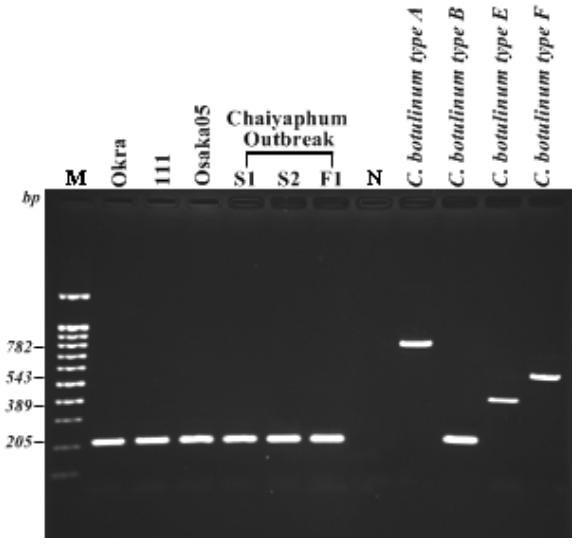

Figure 1. *Sequencing analysis of C. botulinum bont/B genes from Chaiyaphum 2014 strain*

The bont/B8 gene of C. botulinum Chaiyaphum 2014 strain showed the identical to Surat Thani 2012 strain (KC 714045) which classified as subtype B8 (Fig 2). The nucleotide sequences determined in this work were submitted to the NCBI database and designated to be accession numbers KM 0673795

Discussions

The information about botulism outbreak in Thailand showed that sources of outbreaks in 1997, 1998 and 2006 were associated with home-canned bamboo shoot preparations contaminated with *C. botulinum* type A.[20,21] Pork sausage contaminated with *C. botulinum* type A(B) and fermented soy bean and fermented crab contaminated with *C. botulinum* type B8 were previously reported in several outbreaks.[22,23] The BoNT produced by *C. botulinum* showed sequences differences in both within and between serotypes of wide environmental origin, and has geographical distribution. The gene transfer mechanisms are reportedly responsible for such sequence diversity in this species.[2] In this outbreak, Chaiyaphum 2014 strain was identified to be type B8 *C. botulinum* from fermented bamboo shoots samples. For table 1, the toxin was rarely detected in the sera of patients. Woodruff et al studied that the cultures were positive for 51% of stool specimens and toxin testing was positive for 37% of sera.[24] The lethal dose of 0.2-10 ng per kg for various animal including human,[25] due to MBA is still one of the most sensitive to detect BoNT.[26] The main limiting factors or growth of *C. botulinum* in foods are temperature, pH, water activity, redox potential, food preservatives, and competing microorganisms. All of these factors are interrelated and so changing one factor influences the effect of other factors. The interaction of factors may have a positive or negative effect on the inhibition of *C. botulinum.*[27] In this study, even the same lot of fermented bamboo shoots, the factors of the different may be the container contaminated with spore of *C. botulinum* and the random sampling which cause the variation of the results.

More than 90% of the outbreaks are caused by home-prepared or home-preserved foods, especially vegetables (asparagus, green beans and peppers),[28] dairy and fishery products.[29,30] Intoxications with *C. botulinum* producing toxin are often associated with vegetable -based products that at some point contained soil with *C. botulinum* spores.[31] Although heating at 100°C for 10 min can destroy botulinum toxin in food, but the spores are heat-resistant and can survive even after prolonged heating. The thermo-resistance of *C. botulinum* spores varies by strain and other factors such as the lipid and protein content of the food matrix.[31] In this outbreak, the patients did not heat the fermented bamboo shoots before eating. Certain environmental conditions, such as the absence of oxygen (anaerobic conditions), a pH of over 4.6, warm temperatures (greate than 4°C), moisture content (water activity) and a lack of competing bacterial flora could promote the botulinum toxin production in foods contaminated with *C. botulinum.*[32] The alteration of evironmental conditions could increase the pH and allow for the growth of *C. botulinum* and toxin production.

Conclusion

Foodborne botulism remains a public health problem because of its severity and epidemic potential. Our experiental data showed that the etiologic agent of outbreak in Chaiyaphum Province was fermented bamboo shoot contaimated with *C. botulinum* type B8. The molecular methods allow rapid identification and a better understanding the epidemiology of botulism so that proper preventive protocol has to be prioritized at the hospital level. It seems that variation of toxin variants will need to be produced for therapeutics and vaccine. Source of outbreak of foodborne botulism was associated with consumption of home-made, home canned products. Improper cooking may have fostered *C. botulinum* growth and toxin formation. To prevent the future botulism outbreaks, raising awareness of botulism related to home-made fermented food and proper cooking process should be advocated.

Acknowledgements

We thank the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health for the information of clinical and symptom of patients. We thank Dr. T. Ramamurthy, National Institute of Cholera and Enteric Diseases, Kolkata India for his helpful comments on this manuscript.

Reference

1. Arnon SS, Schechter R, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, et al Botulinum toxin as a biological weapon: medical and public health management. JAMA 2001; 285: 1059-70.
2. Hill KK, Smith TJ, Helma CH, Ticknor LO, Foley BT, Svensson RT, et al. Genetic diversity among Botulinum Neurotoxin-producing clostridial strains. J Bacteriol 2007; 189: 818-32.
3. Dover N, Barash JR, Hill KK, Xie G, Arnon SS. Molecular characterization of a novel botulinum neurotoxin type H gene. J Infect Dis. 2014; 209: 192-202.
4. Mazuet C, Sautereau J, Legeay C, Bouchier C, Bouvet P, Popoff MR An atypical outbreak of foodborne botulism due to types B and E from ham. J Clin Microbiol. 2014; Nov 26. pii: JCM.02942-14.
5. Feng L, Chen X, Liu S, Zhou Z, Yang R Two-family outbreak of botulism associated with the consumption of smoked ribs in Sichuan Province, China. Int J Infect Dis. 2014; 30: 74-7.
6. Walton RN, Clemens A, Chung J, Moore S, Wharton D, Haydu L, et al. Outbreak of type E foodborne botulism linked to traditionally prepared salted fish in Ontario, Canada Foodborne Pathog Dis. 2014; 10: 830-4.
7. Takeshi K, Fujinaga Y, Inoue K, Nakajima H, Oguma K, Ueno T, et al. Simple method for detection of Clostridium botulinum type A to F neurotoxin genes by polymerase chain reaction. Microbiol Immunol 1996; 40: 5–11.
8. Nordic Committee on Food Analysis Botulinum toxin. Detection in foods, blood and other test materials. Method no. 79, 2nd ed., Finland: NCFA Espoo. 1991
9. Nevas M, Lindström M, Hielm, Björkroth KJ, Peck MW, Korkeala H. Diversity of proteolytic Clostridium botulinum strains, determined by a pulsed-field gel electrophoresis approach. Appl Environ Microbiol. 2005; 71: 1311-7.
10. Keto-Timonen R, Nevas M, Korkeala H. Efficient DNA fingerprinting of Clostridium botulinum types A, B, E, and F by amplified fragment length polymorphism analysis. Appl Environ Microbiol. 2005; 71: 1148-54.
11. Jacobson MJ, Lin G, Whittam TS, Johnson EA. Phylogenetic analysis of Clostridium botulinum type A by multi-locus sequence typing. Microbiol. 2008; 154: 2408-15.
12. Lindström M, Korkeala H. Laboratory diagnostics of botulism. Clin Microbiol Rev. 2006; 19: 298–314.
13. Aranda E, Rodriguez MM, Asensio MA, Cordoba JJ. Detection of Clostridium botulinum types A, B, E and F in foods by PCR and DNA probe. Lett Appl Microbiol. 1997; 25: 186–90.
14. Hatheway CL. Botulism. In Balows A, Hausler, WH. Ohashi M and Turnano A (eds.), Laboratory diagnosis of infectious disease: Principles and Practice, vol 1. New York: Springer-Verlag 1988; 112-133.
15. Dowell VR Jr, Hawkins TM. Laboratory methods in anaerobic bacteriology CDC Laboratory Manual. Atlanta: DHEW Publication. 1987; 8272.
16. Holdeman LV, Cato EP, Moore WEC. Anaerobe Laboratory Manual 4ed Blacksburg: Virginia Polytechnic Institute and State University 1977; 156.
17. Lindström M, Keto R, Markkula A, Nevas M, Hielm S, Korkeala H. Multiplex PCR assay for detection and identification of Clostridium botulinum types A, B, E, and F in food and fecal material. Appl Environ Microbiol. 2001; 67: 5694-9.
18. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. BMC. 2007; 23: 2947-8.

19. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011; 28: 2731-9
20. Swaddiwudhipong W, Wongwatcharapaiboon P. Foodborne botulism outbreaks following consumption of home-canned bamboo shoots in Northern Thailand. J Med Assoc Thai. 2000; 83: 1021-5.
21. Centers for Disease Control and Prevention. Botulism from home-canned bamboo Shoots -- Nan Province, Thailand, March 2006. MMWR. 2006; 55: 389-92.
22. Wangroongsarb P, Kohda T, Jittaprasartsin, C, Suthivarakom K, Kamthalang T, Umeda K, et al. Molecular characterization of Clostridium botulinum isolates from foodborne outbreaks in Thailand, 2010. PLos One. 2014; 9(1): e77792
23. Wangroongsarb P, Jittaprasartsin C, Suthivarakom K ,Kamthalang T, Yeesoonsang S, Sangkitporn S. An outbreak of foodborne botulism in Surat Thani Province, Thailand, 2012. Jpn J Infet Dis. 2013; 66: 353-4.
24. Woodruff BA, Griffin PM, McCroskey LM, Smart JF, Wainwright RB, Bryant RG, et al. Clinical and laboratory comparison of botulism from toxin types A, B, and E in the United States, 1975-1988. J Infect Dis 1992; 166: 1281-6.
25. Scarlatos A, Welt BA, Cooper BY, Archer D, DeMarse T, Chau KV. Methods for detecting botulinum toxin with applicability to screening foods against biological terrorist attacks. Journal of Food Science 2005; 70: 121-30.
26. Sidell FR, Takafuji ET, Franz DR, eds. Medical aspects of chemical and biological warfare. Department of the Army, Washington, DC: Office of The Surgeon General and Borden Institute. 1997.
27. Centers for Disease Control and Prevention. Botulism in the United States, 1899–1996, handbook for epidemiologists, clinicians and laboratory workers. Atlanta: The Centers. 1998.
28. St Louis ME. Botulism. In: Evans AS, Brachman P, eds. Bacterial infections of humans: Bacteriology and Control. 2nd ed. New York: Plenum Publishing. 1991
29. Lindström M, Myllykoski J, Sivelä S, Korkeala H. Clostridium botulinum in cattle and dairy products. Crit Rev Food Sci Nutr. 2010; 50: 281-304.
30. Aberoumand A. Occurrence of Clostridium botulinum in fish and fishery products in retail trade. World J Fish & Marine Sci. 2010; 2: 246-50.
31. King LA. French Multidisciplinary Outbreak Investigation Team Two severe cases of botulism associated with industrially produced chicken enchiladas, France, August 2008. Euro Surveill. 2008; 13: pii: 18978
32. Loutfy MR, Austin JW, Blanchfield B, Fong IW. An outbreak of foodborne botulism in Ontario. Can J Infect Dis. 2003; 14: 206–9.

Figure legend

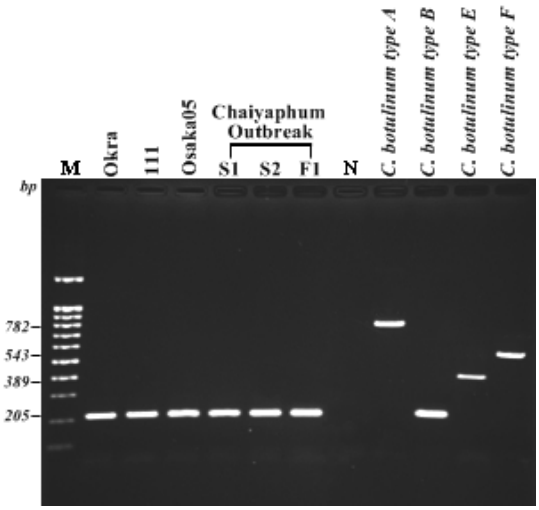

Fig 1. Multiplex PCR for typing of Chaiyaphum 2014 strain.

Lane M and N are 100 bp DNA marker and negative. Lane 1-6 were the bont/B amplicon (205 bps) from strain Okra, 111, Osaka05, Chaiyaphum 2014 (S1, S2 and F1), respectively. Lane 7-10 were the bont/A (782 bps), bont/B (205 bps), bont/E (389 bps) and bont/F (543bps) amplicons from C. botulinum type A (DMST27808), type B (DMST27809), type E (DMST48795) and type F (DMST 27810), respectively.

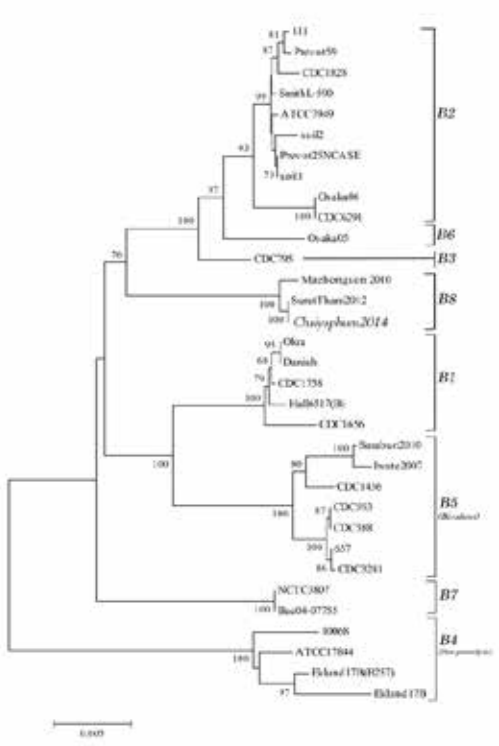

Fig 2. Comparison of the amino acid sequences of bont/B1 to B8 subtypes.

The phylogenetic analysis of nucleotide sequences representing all bont/ B. One representative of each subtype is shown. The phylogenetic tree was generated using the neighbor-joining method and p-distance model. Genetic distance and bootstrap values are shown. Botulinum toxin subtypes, and strains and Genbank accession numbers used in the analysis are as follows: B1 = Okra (AB232927); CDC1758 (EF033127); Danish (M81186); Hall6517(B) (EF028399); CDC1656 (EF028396); B2 = 111 (AB302854); Prevot25 NCASE (EF033129); ATCC7949 (EF028395); Smith L-590 (EF028398); Prevot59 (EF033128); CDC1828 (EF051571); CDC6291 (EF028401); Osaka06 (AB302853); Korean soil 1 (DQ417353); Korean soil 2 (DQ417354); B3= CDC795 (EF28400); B4= EKlund 17B (X71343); 10068 (EF028402); Eklund 17B (B257) (EF051570); ATCC17844 (EF028394); B5= CDC593 (AF300466); CDC1436 (AF295926); 657Ba (EF033130); CDC588 (AF300465); CDC3281 (Y13630); Iwate 2007 (AB665556); Saraburi 2010 (JQ964804); B6=Osaka05 (AB302852); B7 = Bac-04-07755 (JQ354985); NCTC3807 (JN120760); B8 = Maehongson 2010 (JQ964806); Surat Thani 2012 (KC714045); Chaiyaphum 2014 (KM0673795)

TABLE LEGEND

Table 1. Chaiyaphum 2014 outbreaks identified by using culture, mouse bioassays and molecular techniques.

Sample			received date	Mouse bioassay				Isolation	Molecular
				A	B	E	F	C. botulinum	typing
Sera	Patient 1	13-57-06635 (BT)	22 April 57	A	A	A	A	ND	ND
	Patient 2	13-57-06636 (BT)	22 April 57	A	A	A	A	ND	ND
	Patient 3	13-57-06724 (BT)	28 April 57	A	A	A	A	ND	ND
		13-57-06725 (AT)	28 April 57	A	A	A	A	ND	ND
	Patient 4	13-57-06726 (BT)	28 April 57	A	A	A	A	ND	ND
		13-57-06727 (AT)	28 April 57	A	A	A	A	ND	ND
Stool	Patient 1	13-57-06637 (S1)	22 April 57	D	A	D	D	+	B8
	Patient 3	13-57-06728 (S2)	1 May 57	D	A	D	D	+	B8
	Patient 4	13-57-06729	1 May 57	D	A	D	D	+	B8
Fermented bamboo shoots	2 samples	13-57-06653 (F1)	22 April 57	D	A	D	D	+	B8
		13-57-06730	1 May 57	A	A	A	A	-	ND