DEVELOPMENT OF ENZYME-LINKAGE IMMUNOSORBENT ASSAY AGAINST TYPE B OF *CLOSTRIDIUM BOTULINUM*: A PRELIMINARY STUDY

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ABSTRAK

Neurotoksin *Clostridium botulinum* (BoNTs) merupakan salah satu penyebab kerugian ekonomi bagi industri peternakan. Kerugian ini dapat sebagai akibat langsung ketika ternak keracunan BoNTs atau secara tidak langsung ketika produk asal ternak terkontaminasi BoNTs sehingga produk tersebut dilarang untuk dipasarkan. Untuk itu perlu dilakukan pengontrolan rutin penyebaran BoNTs di lingkungan peternakan dan industri hasil ternak. Salah satu caranya adalah dengan melakukan deteksi rutin terhadap keberadaan BoNTs. Salah satu metode deteksi yang relatif cepat dan akurat adalah metode enzyme linkage immunosorbant assay (ELISA). Pada penelitian ini dilaporkan hasil pengembangan ELISA, menggunakan antibodi poliklonal produk lokal terhadap BoNTs-B. Antibodi dihasilkan dari enam mencit Balb/c dengan metode imunologi standar. Mencit diimunisasi 3 kali selama 8 minggu dengan toksoid C. Botulinum tipe B komersial dengan dosis 100 ng per ekor per injeksi. Antibodi yang dihasilkan dimurnikan dengan kombinasi metode presipitasi ammonium sulfat 50% (w/v) dan kolom protein A. Hasil studi pendahuluan ini menunjukkan bahwa metode ELISA yang dikembangkan mampu mendeteksi toksin *Clostridium botulinum* tipe B hingga 1,0 ng/ml.
Kata kunci: toksin C. botulinum, ELISA, antibodi poliklonal

ABSTRACT

Clostridium botulinum neurotoxin (BoNTs) is one of the causes of economic loss in the livestock industry. This economic loss would be as a direct result when animals poisoned by BoNTs or indirectly when the livestock products are contaminated by BoNTs, which end up with the products are banned by authority. Therefore a routine surveillance of BoNTs in the farm and in livestock product processing industry is urgently needed. One of the most relatively quick and accurate methods to perform a routine detection of the presence of BoNTs is enzyme-linkage immunosorbant assay (ELISA). In this article we describe the results of the development of ELISA, using polyclonal antibodies against BoNTs-B produced locally. Antibodies were generated from six Balb/c mice with standard immunological methods. Mice were immunized three times for a period of 8 weeks with a commercial type B *Clostridium botulinum* toxoid at a dose of 100 ng per mouse per injection. The resulting antibody was purified by a combination of ammonium sulfate precipitation 50% (w/v) technique and a protein A column method. The results of this preliminary study indicated that the developed ELISA method capable of detecting type B *Clostridium botulinum* toxin up to 1.0 ng/ml.
Keywords: C. botulinum toxin, ELISA, polyclonal antibody.

INTRODUCTION

Clostridium botulinum neurotoxin (BoNTs) is the causative agent of botulism, a fatal disease characterized by muscular paralysis, which affect mammals, birds, and fish (Keto-Timonen *et al*., 2005, Steinman *et al*., 2006). Livestock botulism is a worldwide problem that causes large economic losses (Steinman *et al*., 2006). The losses that incurred on the farm industry can be happened directly or indirectly. The direct loss occurred when the animal is attacked directly, such as when they ingest *C. botulinum* spores contaminated feed. Indirect losses arose if the spores are found in animal processed products such as sausages or burgers (Shapiro *et al*., 1998),

even in honey (Nevas *et al*., 2002), which in turn causes the products are no longer economically valuable even banned by the authority. To overcome the problems early prevention is needed. This can be done by both routine handling of the livestock sanitary as well as maintaining the hygiene of processing livestock products. In addition routine screening tests again BoNTs are required.

Commonly test method performed is mouse bioassay, which measures the resistance of mice to the toxin (Witcome *et al.,* 1999; Gessler *et al*., 2005). The assay is able to detect up to one unit dose of one mouse lethal dose (1 MLD), which is equivalent to approximately 10 pg/ml of neurotoxins (Sharma *et al*., 2005). Hence, this method is categorized quite sensitive. However, the operational cost of this method is relatively high due to the need of mice with specific conditions. Furthermore it takes special skills, animal ethics concerns, as well as the specificity of neurotoxins detected is still very limited. It also needs quite long time to get results, which is about 4 days or more (Sharma *et al*., 2005). Consequently several methods have been developed ranging from ELISA (Dezfulian *et al*., 1984; Witcome *et al*., 1999), immunoaffinity chromatographic column test (Gessler *et al*., 2005), fluorogenic assay (Anne *et al*., 2001), up to the bio-molecular test such as PCR (Fach *et al*., 1995; Braconnier *et al*., 2001; Kimura *et al*., 2001; Lindstrom *et al*., 2001; Fach *et al*., 2002).

The advantages of the ELISA method are faster than the mouse bioassay method, sensitive with high specificity, easy to standardize, and is commonly performed even to the level of public health laboratories (Sotelo *et al*., 2011). In the present study it was attempted to develop an ELISA method to the *C. botulinum* toxin using the antibody which was developed locally. The results should provide a better understanding of the development of the method as well as to carry out an early protection against the spreading of *C. botulinum* in the farm and animal industries.

MATERIALS AND METHODS

Polyclonal Antibody Production

Polyclonal antibodies used in this study were generating locally by Depamede and Kisworo (2009). Polyclonal antibodies were produced in mice based on Dixit *et al.,* (2006) and Keller *et al.,* (2008) with some modifications as mentioned by Depamede and Kisworo (2009). Six female

Balb/c mice aged 6-8 weeks were immunized with a commercial type B *C. botulinum* toxoid (Lot No. B121908-01, Metabiologics, Inc., U.S). Mice were immunized 3 times for 8 weeks with a dose of 100 ng per mouse with 4 weeks interval. In the initial immunization, toxoid was mixed with complete Freund's adjuvant (CFA, Sigma) to form an emulsion. On the second immunization a mixture with incomplete Freund's adjuvant (ICFA, Sigma) was performed. The first and the second injection were carried out subcutaneously, while the third injection (at week 8) was administered intramuscularly. As a control, Balb/c mice were injected concurrently with the treatments but the toxoid was replaced with physiological saline solution. Fourteen days after final immunization, serum of each treatment group and control was taken from the mouse tail. The serum was then frozen at -20°C until processed for purification.

Isolation and Purification of Polyclonal Antibodies

Isolation and purification of polyclonal antibodies against BoNTs-B toxin generated from Balb/c mice was carried out by 50% (w/v) ammonium sulfate precipitation and Protein A column methods (Baumgarten, 1992). The purity of the antibody was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Hempelmann (1992).

Development of ELISA

The isolated antibodies and a commercial antibody (Abcam) were used to develop sandwich ELISA according to Farzamfar *et al.* (2007) with slight modification as performed by Depamede and Kisworo (2009). ELISA plates were firstly coated with rabbit anti type B (RaCB) *C. botulinum* toxin commercial (Abcam) as the first antibody layer. Each well was coated with 100 μL (2 μg/ml) antibody in carbonate buffer solution pH 9.5 and then allowed to stand at 4°C for 12 hours. After that the solution was discarded, replaced with 300 μL blocking solution (PBS containing 0.05% (v/v) Tween 20 (Sigma), and 0.5% (w/v) bovine serum albumin) and allowed to stand for 60 minutes at room temperature. The plates were then washed five times with 300 μL/well of washing solution (PBS containing 0.05% (v/v) Tween 20). After washing, the wells were filled with 50 μL/well of standard commercial ELISA for *C. botulinum* toxin (Metabiologics, Inc.) with a concentration of 100

ng, 1 ng, 0,2 ng, 0.1 ng, and 0 ng/ml, and incubated for 60 min at room temperature. Then the plate was washed again 5 times and then 50 µL of polyclonal antibody generated in this study as the second layer, concurrently with the controls was added in to the wells and incubated for 60 minutes at room temperature. The purpose of these second layer antibodies are as a linker between suspected antigen (BoNTs) in the samples and the enzyme Horseradish peroxidase(HRP)-conjugated antibody. After washing, 50 µL goat-anti-mouse IgG HRP-conjugated antibodies were dropped into the ELISA plate wells and incubated again for another 60 minutes at room temperature. After the last wash, a 50 µL substrate was added to the wells and incubated for 5-10 minutes in the dark, at room temperature until the reaction color changes in the positive samples occurred. The reaction was stopped by adding 50 µL of 1 N H_2SO_4 then the reaction products were quantified by ELISA photo reader (DYNEX-MRX) on the optical density of 450 nm (OD 450).

Data Analysis

The data obtained were tabulated and analyzed by a simple arithmetic mean (Mean ± SD) of the OD 450 (Drouet *et al.,* 2003) and presented as reported by Depamede and Kisworo (2009). The calculation of the limit value of positive or negative test results (cut-off value, COV) is calculated based on Drouet *et al.* (2003), ie, COV = average value of negative controls ± 3 x SD of negative control value. Samples with a COV ≤ OD 450 value was designated negative, while a greater than COV was positive (Depamede and Kisworo, 2009).

RESULTS AND DISCUSSION

Of the six Balb/c mice used, one animal died during the study due to unknown reasons, but certainly not as a result of vaccination. Of the 5 mice leaved, 4 (80%) gave a positive immune response or produced polyclonal antibodies against type B *C. botulinum* toxin, and one of them gave a quite high immune response, while one was with the lowest immune response (+/-) as presented in Table 1.

From these results it appears that the immune response of Balb/c mice was individually quite disperse (variation value was about 47.63%), and only one mouse gave high enough immune response. These results were consistent with previous studies, especially when the vaccine or immunogen used was a commercial immunogen; native immunogen reported to be more immunogenic than those of the commercial toxoid (Witcome *et al.,* 1999; Keller, 2008). In the present study, immunogen used was a commercial *C. botulinum* toxoid. Technically, the use of commercial immunogen is more secure than the intact or native immunogen derived or isolated directly from *C. botulinum*.

Furthermore, from the results of this study as reported also by Depamede and Kisworo (2009), it was noted also that the dose of 100 ng/ml gave a significant immune response (p<0.05) on the production of polyclonal antibodies (Figure 1). Polyclonal antibodies obtained were then purified by ammonium sulfate precipitation method and the protein A 'home made mini-column'. The results of purification were analyzed using SDS-PAGE as shown in Figure 1. From this figure, it clear knwn that the antibody obtained was quite pure.

Polyclonal antibody obtained was then used as a component of the development of indirect ELISA as mentioned in the mateials and methods or as explained elsewhere by Depamede and Kisworo (2009). Preliminary results of the developed ELISA method in this study showed

Table 1. Immune Response of Balb/c mice Against *C. botulinum* Toxoid

Balb/c Code	The mean value OD$_{450}$(n=3)	Immune Response (COV=0.350)
1	0.580	+
2	1.156	+++
3	†)	†)
4	0.423	+/-
5	0.500	+
6	0.642	+

†) *Mouse died during the study*
(Source: Depamede and Kisworo, 2009).
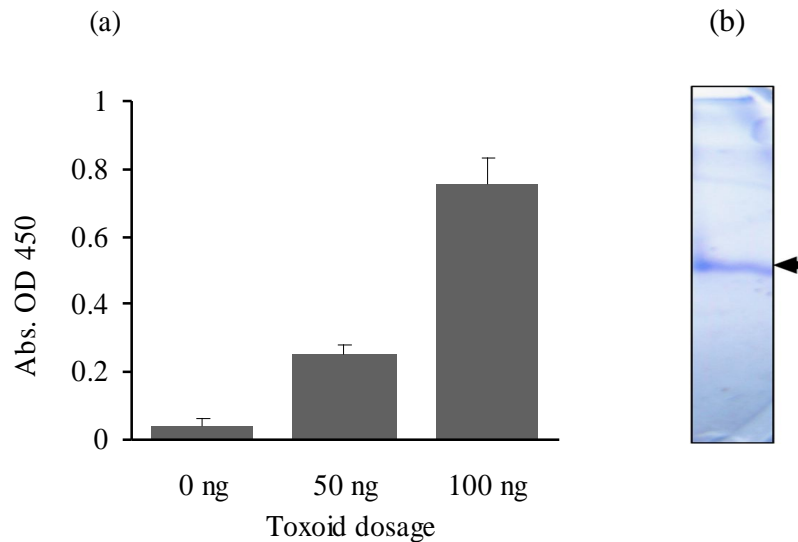
(a)   (b)

Figure 1. Effects of a Commercial *C. botulinum* Toxoid Vaccine Dosages on Production of Polyclonal Antibodies of Local Balb/c Mice (**a**) and a Representative Result of 12.5% SDS-PAGE Analysis of Polyclonal Antibodies (arrow) Purified by Protein A Column (**b**).

that polyclonal antibodies generated in this study can be used as a component of ingredients needed to develop immunoassay such as ELISA against *C. botulinum* toxin. Figure 2 shows that the developed ELISA was able to detect type B *C. botulinum* toxin up to a concentration of 1.0 ng/ml.

When compared to the *in vivo* mouse bio-assay test, the results of this preliminary study were yet as sensitive as the bio-assay method. Bio-assay method was able to detect the toxin of *C. botulinum* from 0.01 up to 0.02 ng (Witcome *et al.,* 1999). Although the bio-assay method is more sensitive, as mentioned elsewhere, the method requires animals with special handling, special skills, and time consume. Furthermore, there is a consideration of restrictions on the use of trial animals that increasingly constricted in the last few decades (Witcome *et al.,* 1999). These considerations have led the researchers to develop a more practical method of testing by applying the principles of immunodiagnostics such as ELISA.

Lee *et al.* (2005), Atassi (2006), and Keller (2008) suggested that if immunodiagnostics need to be developed, it would be better if using antibodies produced from modified *C. botulinum*-intact toxin. They found that "home-made vaccine" from native *C. botulinum* antigens were more immunogenic and more protective than those of commercial antigens (Keller, 2008). Furthermore, Witcome *et al.* (1999) has
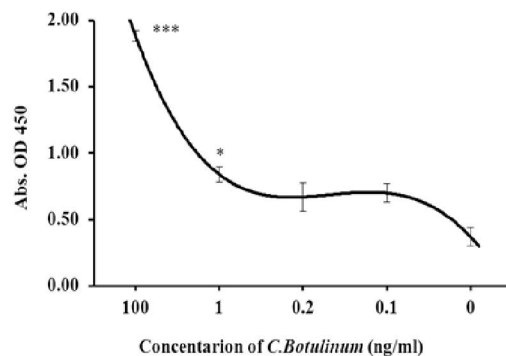

Figure 2. The Results of the Detection of a Commercial *C. botulinum* toxoid (BoNT) using the ELISA Developed in this Study. Toxin was Detected up to 1 ng/ml. ** $P < 0.001$; * $P < 0.05$ Compared to Control (0 ng/ml, n = 3).

successfully developed a modified ELISA test using monoclonal antibodies produced from the modified native *C. botulinum* antigens. It was claimed that the modified ELISA was able to detect the *C. botulinum* toxin up to 0.005 ng, or in other words two times more sensitive than the bio-assay method, which was able to detect 0.01 ng (Witcome *et al.,* 1999). From this account, it can be revealed here that the quality of the antibodies obtained in this preliminary study to be used as component for the development of ELISA

can still be improved further.

CONCLUSIONS

An ELISA kit for detecting *C. botulinum* toxins has been developed by applying polyclonal antibodies against *C. botulinum* generated locally in this preliminary study.

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