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Development of ELISA and colloidal gold immunoassay for tetrodotoxin detetcion based on monoclonal antibody

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ABSTRACT

A monoclonal hybridoma cell named 5B9 against tetrodotoxin (TTX) was obtained after fusion of myeloma SP2/0 cells with spleen cells isolated from the immunized Balb/c mice. The 5B9 monoclonal antibody (McAb) with high affinity (about 2.55×10^9) is specific to TTX, and this McAb belongs to the immunoglobulin G (IgG) isotype. Finally, an enzyme-linked immunosorbent assay (ELISA) and colloidal gold immunoassay were established based on this McAb. The linear range of ELISA to detect TTX was 5–500 ng/mL, and the limit of detection (LOD) was 4.44 ng/mL. The average CV of intra- and inter-assay was less than 8%, with the samples recovery range of 70.93–99.99%. A competitive format colloidal gold strip was developed for detection of TTX in real samples, and the LOD for TTX is 20 ng/mL, and the assay time of the qualitative test can be finished in less than 10 min without any equipment. The result from test strip revealed that the test strip has a good agreement with those obtained from ELISA.

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1. Introduction

Tetrodotoxin (TTX) is a small molecular and non-protein marine toxin, and mainly exists in the nearly 100 species of puffer fish, especially concentrated in the ovaries and liver, following in the blood, eyes and skin of puffer fish (Liu et al., 2011; Narahashi et al., 1967). TTX is a highly toxic neurotoxin and only 0.5–3 mg of it can make an adult die of poisoning, and the median lethal dose (LD_{50}) for mice is 80 μg/kg by intraperitoneal injection. TTX can also combine with the receptors of sodium ion channel on the nerve cell membrane, resulted in nerve paralysis, respiratory failure and eventual death (Narahashi et al., 1967; Kao, 1966). Every year, many poisoning cases were reported in coastal areas owe to consumption of delicious puffer fish. Besides, TTX is stable and not easy to be destroyed only in the cooking condition. Therefore, development of a fast and quantitative analysis for detection of TTX in real samples is getting more and more important.

At present, some traditional methods based on different principle for TTX detection were developed, including mouse bioassay, high performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS), thin-layer chromatography, and so on. (Strong et al., 1973; O'Learya et al., 2004; Kazuyuki and Susumu, 1980; Ikebuchi et al., 1988; Ho et al., 1994). These traditional methods are often restricted due to low-level efficiency or lacking of practicality. For example, mouse bioassay is time-consuming and poorly precision (Mebs et al., 1995), and HPLC analysis often needs expensive equipment, and the process of sample handling was tedious (Yotsu, 1987). The detection method based on immunoassay is rapid, sensitive, and economic, and was widely used to detect pathogens (Watabe et al., 1989). Enzyme-linked immunosorbent assay (ELISA) is the main assay method among the immunoassay. Monoclonal antibody (McAb) with high specificity and affinity has been widely used in ELISA, radioimmunoassay, immunohistochemistry and flow cytometry experiments. Tao et al. (2010) reported that a competitive indirect ELISA was developed for detection of tetrodotoxin, and the limit of detection (LOD) was 5 ng/mL. Stokes et al. (2012) reported that an improved competitive inhibition enzymatic immunoassay method was used for TTX determination, and the LOD was 10 ng/mL. In the process of ELISA, several steps of binding, wash and coloration are often needed, so the total time for detection was longer. Compared to the traditional ELISA, the developed colloidal gold nanoparticle probe for the immunoassay is rapid and accuracy, and the

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detection can be finished in several minutes. Zhou et al. (2010) used gold nanoparticle probe-based immunoassay as a new tool for tetrodotoxin detection in puffer fish tissues, and the visual LOD of TTX-spiked samples was found to be 40 ng/mL. The assay time of the qualitative test based on this visual evaluation for TTX detection was less than 10 min.

In this study, we aim to prepare an McAb with high affinity and specificity against TTX, and to establish ELISA and colloidal gold immunoassay methods for TTX detection based on the McAb. TTX is a small molecular hapten without immunogenicity, so the TTX–Bovine serum albumin (TTX–BSA) and TTX–Keyhole limpet hemocyanin (TTX–KLH) conjugates were used as the detection and immunogen antigen, respectively. Finally, a IgG McAb with high titer against TTX was obtained, and the ELISA with high sensitivity and reproducibility was developed to detection TTX. The preparation of a colloidal gold nanoparticle probe and the development of a colloidal gold immunoassay for the rapid detection of TTX were also carried out.

2. Materials and methods

2.1. Production of monoclonal antibody

All the needed materials were in Supporting infromation. Three female Balb/c mice (6–8 weeks old) were subcutaneous injected respectively with antigen TTX–KLH. After 4 times injection, the titer of antibody from the immunized mouse was monitored by indirect ELISA (iELISA). Spleen cells were isolated and collected from the immunized mice, and SP2/0 myeloma cells were mixed with the spleen cells at ratio of 1:10 and fused in 1 mL 50% PEG. The hybridoma was subcloned by limiting dilution method (Davis et al., 1982). McAb was prepared by the method of introduced ascites in abdomen (Groopman et al., 1984). The IgG from the ascites fluid was purified by ammonium sulfate precipitation, and then stored at −20 °C for analysis. More details were in Supporting information.

2.2. Characterization of hybridam antibody

Chromosomes of cells were analyzed according to the modified method (Freshney, 1983). IELISA was used to determine the titer of IgG from the ascites fluid. SDS-PAGE was used to determine the purification of IgG McAb (Laemmli, 1970). The isotype of resulting antibodies were determined according to the instructions for the isotype kit. According to the method provided by Beatty et al. (1987), the affinity constant *Kaff* of McAb was determinated by iELISA (both coated antigen and McAb were doubling diluted). The specificity of antibody was detected by indirect competitive ELISA (icELISA). The concentration at IC50 was used to calculate cross-reaction rate. More details were in SI.

2.3. iELISA for tetrodotoxin detection

The repeatability and accuracy of iELISA were determined by detecting the coefficient variation (CV) of intra- and inter- assay, and the recovery of sample, respectively. After different concentrations of TTX standard were added, samples were detected to calculate the average recovery. Pretreatment of real samples were developed according to the procedures in literature with some modification (Zhou et al., 2010). More details were in Supporting information**.** When really samples were assayed, the samples were detected by icELISA after treatment with acetic acid, and the TTX concentration of sample was calculated according to standard curve.

2.4. Specificity and sensitivity of strip test

Preparation of colloid gold strip was carried out as mentioned in Supporting information**.** To evaluate the specificity of the test strip (Wang et al., 2014), different toxins including Microcystin (MC), Saxitoxin acetate (STX), Conotoxin (CTX), OkadaicAcid (OA), Pectenotoxin (PTX) were allowed to react with the colloidal gold–TTX McAb conjugate which was pipetted into glass fiber paper. The mixture then moves upward on the nitrocellulose membrane. After incubation at room temperature for 5 min, the detection results could be observed by the naked eye. For the sensitivity test, the series concentrations of 0–80 ng/mL toxins were allowed to react with the colloidal gold–TTX McAb conjugates. The sensitivity and LOD for TTX can be evaluated according to the result from colloid gold strip.

2.5. Assay of TTX in samples with colloid gold strip test

Five different kinds of actual samples from Fujian province in China were detected by colloidal gold strip test. Pretreatment of real samples were developed according to the procedures in literature with some modification (Zhou et al., 2010). More details were in Supporting information. Subsequently, the extracted sample solution was migrated on the membrane, and the strip test was allowed to develope red color for 5–10 min. The detection results could be evaluated according to the result from colloid gold strip.

3. Results

3.1. Screening of monoclonal antibody

Mice immunization was carried out by injecting antigen TTX–KLH into 3 mice for 5 times, and the serum titer was determined by iELISA. The result was in Fig. S1A. Mice 1 had the highest titer, and it was chosen to do the further fusion experiment for preparing monoclonal antibody against TTX. TTX was a typical hapten with molecular weight of 319, so it cannot induce immune response itself. In the study, TTX–KLH conjugate as an immunogen was used to immune mice, and the satisfactory result (Fig. S1A) showed that the TTX–KLH could effectively stimulate the body to produce antibodies against TTX after the 5th immunization. From our experiences, immune method was taken by multi-point injection subcutaneously for better immunization effect.

After immunization, spleen cells and SP2/0 cells were fused by PEG, and iELISA was used for screening hybridoma against TTX. Finally, five different hybridomas with higher titer named 1D3, 2E12, 5B9, 5E5, and 6F9, were obtained (Fig. S1B). Screening of McAb lies in the positive hybridoma obtained after fusion and cells subcloning (Rocke and Jones, 1997). The earlier to subclone hybridoma in positive wells, the more chance to get the monoclonal hybridoma. Hybridoma colonies with high titer and good condition should be selected for subcloning. Feeder layer cells were essential for cells fusion and subclone screening, not only because it could secrete a variety of growth factors to support the growth of hybridoma, but also could clean up cell debris by phagocytosis (Selby, 1999).

3.2. Characterization of hybridoma and McAb against TTX

The average chromosome numbers of SP2/0 myeloma cell and spleen cell were 62–70 and 38–40, respectively. The result showed that there were 102 ± 6 chromosome numbers in one hybridoma (Fig. 1A), matching the anticipated chromosome numbers of hybridoma. The subtype of positive clones 1D3, 2E12, 5E5, 5B9, and

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