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Immuno-fluorescence detection of snake venom by using single bead as the assay platform

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By using micro-scale polystyrene beads as a platform, a rapid and sensitive method for the detection of snake venom was established. In the method, anti-venom antibody or BSA was covalently fixed onto the microsbead to form the capture-bead or control bead. In first step of the experiment, the venom binds to the capture-bead to form the complex through the antibody-antigen interaction. The Qdot conjugated second antibody was then added. The second antibody targeted the Qdot to the capture-bead/antigen complex and form Qdot-second antibody-antigen-capture-bead complex. This complex can be directly observed under UV-microscope. The system was applied to the testing of *Naja kaouthia* venom and the detection limit of this method was 5–10 ng/ml.

Keywords: snake venom; single bead; Qdot; immunoassay

1. Introduction

Antivenom therapy has proved to be the best choice to deal with envenomation by venomous snakes [1], which remains the major cause of mortality and morbidity in developing countries [2]. Snake venom is mainly composed of toxic proteins and small peptides, which induce diverse clinical features that require different treatments. Therefore, the key step in antivenom therapy is to identify the venomous snake inflicting the bite and administer the patient with specific monovalent antivenom [3]. Because of its specificity, a smaller amount is needed compared with polyvalent antivenom, and administration with monovalent antivenom will reduce the chance of severe allergic reactions and serum sickness resulting from antivenom therapy.

ELISA has become the most widely used method for snake venom detection since it was first introduced by Theakston et al. to detect the snake venom in sera from experimental animals and human victims [4]. The disadvantages of ELISA, particularly

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time and sample consuming, encourage the scientist to explore new immunoassay methods for the diagnosis of snake venom. Numerous reports on detection of snake venoms have been published and these techniques have been extensively reviewed [5–9]. Using particles as an assay platform is one way to replace conventional ELISA and bead-based flow cytometric immunoassays were first reported in 1982 to quantify the human antibody [10]. By using beads preconjugated to capture antibodies, the method completely removed the time for antibody immobilisation. In addition, the method also avoided the step to remove the unbound dye or chemical from the assay as in conventional ELISA. But the method needs the flow cytometry to separate and assay the signal attached on the single bead. In this communication, we present a simple and rapid method: single bead-based immunoassay for snake venom detection.

2. Materials and methods

2.1. *Materials*

The venom of *Naja kaouthia* was bought from Venom Supplier (Australia). The Polybead Carboxylate Microsphere (6 μm) was bought from Polysciences Inc. (Polysciences Inc., Pennsylvania USA). Qdot® 565 streptavidin conjugate was purchased from Invitrogen (Invitrogen, USA). All other chemicals and reagents used were of analytical grade.

2.2. *Protein estimation*

Proteins were determined by Bradford's dye binding method [11]. Bovine immunoglobulin was used as standard for antibody estimation and bovine serum albumin as standard for other protein estimations.

2.3. *Biotinylation of immunoglobulins*

The biotinylation was carried out as follows [12]: Antibodies (1.0 mg/ml) were disolved in 0.1 M sodium bicarbonate buffer, pH 8.3. For 1 ml IgG solution (1 mg/ml), 200 μl of Biotin-*N*-hydroxysuccinimide ester (0.2 mg/ml in DMSO) was added and incubated for 4-h at room temperature. The mixture was then extensively dialysed against PBS, pH 7.4 buffer.

2.4. *Coupling of proteins to the Polybead Carboxylate Microspheres*

Purified IgG or BSA was conjugated to the Polybead Carboxylate Microspheres using Carbodiimide Kit for Carboxylated Microparticles (Polysciences Inc., Pennsylvania USA) following the manufacture's instruction.

2.5. *Bead-based ELISA*

IgG or BSA preconjugated beads (5 μl of 2.5% suspension) were pelleted and the supernatant was discarded. The beads were then suspended in different concentrations

of toxin (dissolved in PBS/Tween solution containing 2% BSA) and incubated at room temperature for 1 hour with gentle mixing. After incubation, the beads were pelleted and washed with PBS/Tween for 3–5 times. The beads were again resuspended with biotinylated antibody solution (1:100 in PBS/Tween solution containing 2% BSA) and incubated for 1 hour with gentle mixing. At the end of the incubation, the beads were pelleted and washed with PBS/Tween for 3–5 times. The beads were then resuspended with avidin-HRP solution (1:1000 in PBS/Tween) for 5 min and again washed with PBS/Tween for 3–5 times. The activity of bead-bind HRP was then assayed.

2.6. *Single bead-based immunoassay*

Testing beads (IgG preconjugated, blue dyed) were first mixed with control beads (BSA preconjugated, red dyed) and suspended in different concentrations of toxin (in 2% BSA PBS/Tween solution) with 1.5 ml testing tubes. The samples were then incubated at room temperature for 1 hour with gentle mixing. After incubation, the beads were pelleted and washed with PBS/Tween three times. The beads were again resuspended with biotinylated antibody solution (1:100 in 2% BSA PBS/Tween solution) and incubated for 1 hour with gentle mixing. At the end of the incubation, the beads were pelleted and washed with PBS/Tween three times. The beads were then resuspended with avidin-Qdot solution (1:100 in PBS/Tween) for 30 min and washed with PBS/Tween three times. The beads were finally suspended in 20 μl of PBS/Tween. The bead suspension was immediately put on a glass slide and checked under a microscope. The density of fluorescence attached to the single bead was analysed by Image J software.

3. Results

3.1. *Procedure of single bead-based immuno-fluorescence assay*

The procedure of single bead-based immuno-fluorescence is outlined in Figure 1. It could be divided into 3 steps: (1) Immobilisation of anti-venom antibody. The capture antibody was covalently conjugated to the microbeads following the procedure described in the methods to produce the capture beads. (2) Antigen-enrichment. During incubation, the capture beads could "catch" the toxin in the solution through antigen-antibody interaction and immobilise it on the bead surface. (3) Antigen detection. To detect the toxin, secondary antibody (biotin-labeled) was added. It binded to the toxin which was immobilised on the capture beads and formed a complex. The presence of the complex was then checked by adding avidin-labeled Qdot and observed under UV/light microscope.

3.2. *Bead immobilisation of the IgG*

In our experiment, anti-*naja kaouthia* venom antibody and BSA were conjugated to blue and red dyed beads separately to form the capture and control beads. 1 ml of the beads could immobilise 1 mg of the protein. After conjugation, the IgG-conjugated beads were then suspended in the storage solution and tested under −20°C. Compared with the freshly prepared beads, the beads stored in −20°C did not show significant changes even

after 1 month. Figure 2 shows the comparison of newly prepared IgG-beads with the one stored in −20°C for 1 month.

3.3. *Bead-based ELISA*

As we are trying to establish the bead-based assay to replace the conventional ELISA, we first compared the sensitivities of both methods. As shown in Figure 3, the testing limit of bead-based ELISA could reach 1–5 ng/ml which was slightly higher than that of conventional ELISA (5–10 ng/ml).

3.4. *Single bead based fluorescence immunoassay*

As shown in Figure 4, only the fluorescence (green) can be seen for the capture-beads under UV (Figure 4(b)) while, the capture-beads (blue) and control beads (red) can be clearly differentiated under visible light (Figure 4(a)). The enlarged area of Figure 4(b) clearly showed that the green fluorescence (from Qdot) evenly distributed on the surface of the capture-beads (Figure 4(c)), indicating that the capture antibody was evenly conjugated on the surface of the beads and the antibody retained its activity to recognise their specific target.

The testing limit of this method was also been tested. Different concentrations of the venom solution were applied in the assay. After taking the photo, the fluorescence intensity of the capture-beads were analysed by Image J software. As shown in Figure 5, the fluorescence gradually increased following the change of venom concentration. Significant change can be seen when the venom concentration reached 5–10 ng/ml when compared with the control.

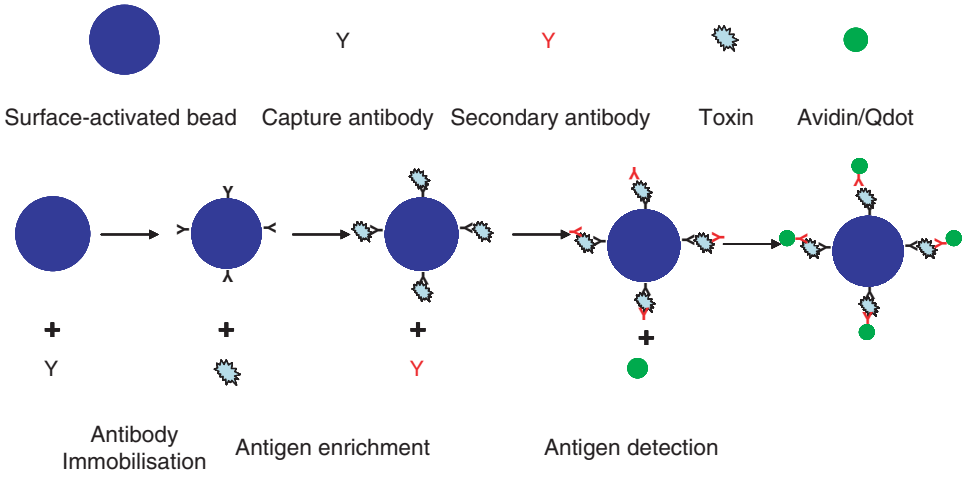

Figure 1. Procedure for single-based immuno-fluorescence assay. The capture beads "catch" the antigen and immobilise the antigen from the solution through the capture antibody its surface. The beads were then washed and incubated with second antibody (biotin labeled). After forming the capture bead-antigen-second antibody complex, avidin/Qdot was added to label the complex through biotin-avidin interaction. The presence of the complex could be checked by UV microscope.

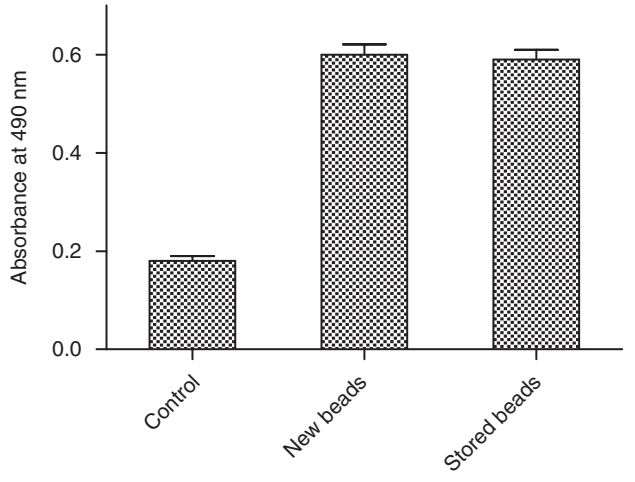

Figure 2. Stability of stored anti-*Naja kaouthia* IgG conjugated beads. Freshly prepared beads and the one stored in−20°C were used to test their capacity to bind the antigen (1 μg/ml) as described in the methods. The histo-gram shows the absorbance of the supernatant was measured at 490 nm for newly prepared (new beads) and −20°C (stored beads) stored beads.

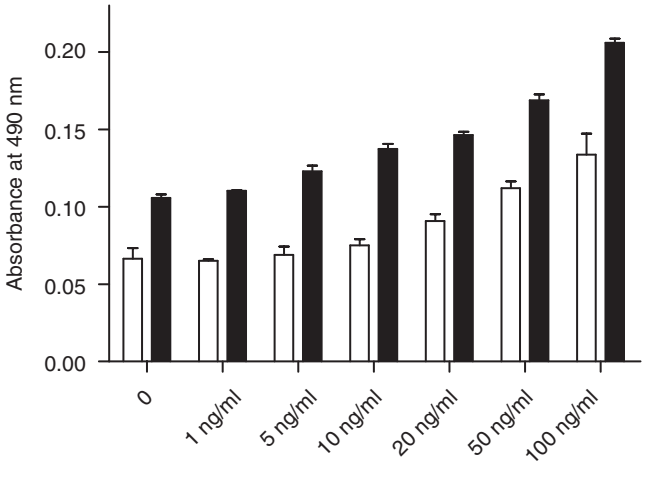

Figure 3. Test limit of conventional and bead-based ELISA. Conventional ELISA testing of *Naja kaouthia* venom was done by using 96-well plate and compared with bead-based ELISA. Empty Bar shows the reading for Conventional ELISA while solid bar for the bead-based ELISA.

4. Discussion

Rapidity, simplicity and sensitivity are the key elements in the development of snake venom detection method. These will help to identify the snake species and administration to the patients of specific antivenom within a short time. ELISA has long been accepted and widely used for the identification of snake species inflicting the bite (4). However, its

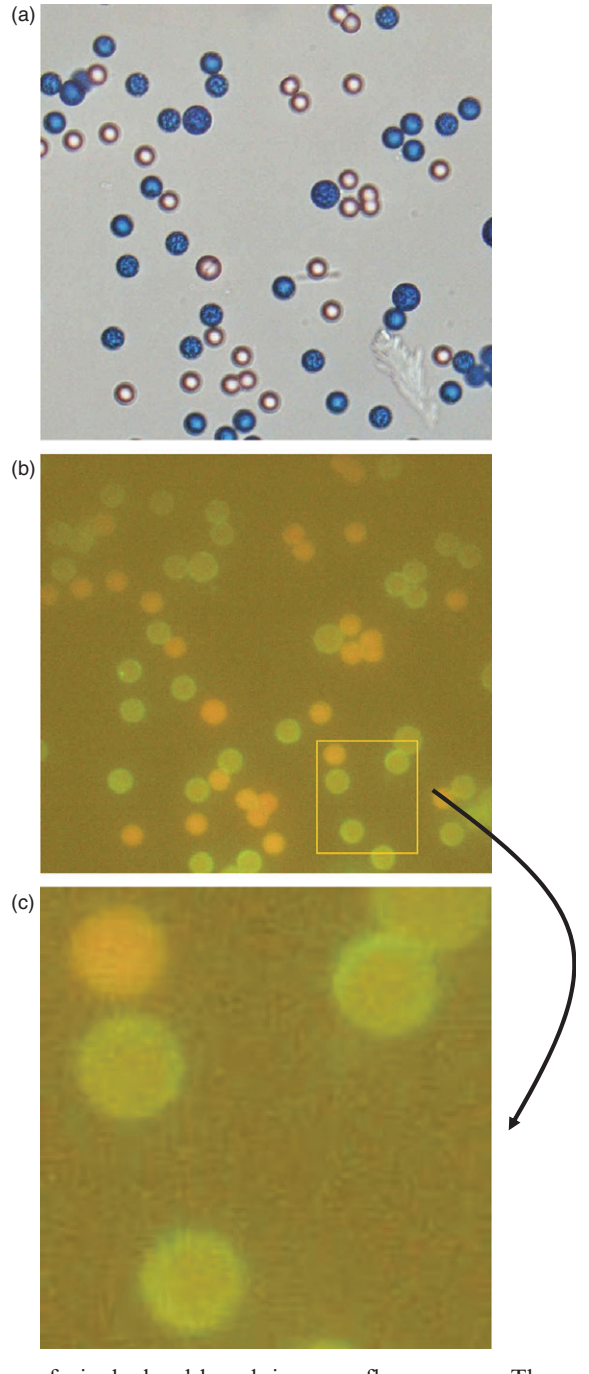

Figure 4. Bead image of single bead-based immuno-fluorescence. The concentration of *Naja kaouthia* used for testing was 1 mg/ml. (a) and (b) showed the image taken under visible and UV light separately. Red dyed beads (control) and blue dyed beads (testing) can be seen under visible light (a) while the green fluoresce can only be seen for the testing beads under UV light (b). (c) shows the enlarged area of image (b).

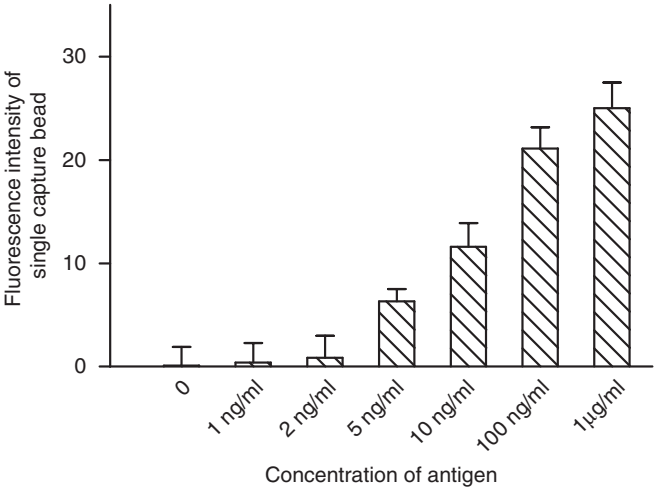

Figure 5. Fluorescence intensity bind to the capture beads. The intensity of fluorescence attached to the single capture bead was quantified by Image J software. Significant change can be observed following the increase of venom concentration.

being time-consuming is one of the major shortcomings for the detection of ELISA. In this method, immobilisation of anti-body is the most time consuming step, which will need at least 12 hours for the coating of microtitre wells. Therefore, long-term stored solid-face conjugated and ready to use antibody is the best way to solve the problem. In the present study, we employed the microsphere as a solid phase to carry out the antigen-antibody reaction. The bead-based technique has been used in Multiplexed Fluorescent Microsphere Immunoassay [13] for the detection of various kinds of antigen and antibody, such as bacteria toxin [14], autoantigens in the serum of the patients [15] and specific antibodies against viral antigens [16]. The nature of the beads makes it possible to carry out immunoassay in a test tube and normal methods, such as stirring and votexing, can also be applied in both antibody immobilisation and antibody/antigen reaction. These will significantly increase the efficiency of antibody immobilisation antibody-antigen interaction when compared with planar surface used in conventional ELISA. In our experiment, microbeads (dyed, 6 μm) were used to covalently conjugate the capture antibody and the pre-conjugated beads could be stored in for long-term use. In doing this, we completely avoid the antibody immobilisation step in conventional ELISA and significantly decrease the time in venom detection.

Besides time, sensitivity is also a key factor in the diagnosis of snake envenomation. Many methods, such as label secondary antibody with fluorescence to replace HRP; have been developed to improve the sensitivity. In our experiment, Qdot was used to label the secondary antibody to detect the captured antigen. The Qdot provides stronger photobleaching resistant fluorescence which could be easily checked under UV microscope, when compared with conventional organic fluorescence dyes. Using our testing system, as little as 5–10 ng/ml of the snake venom solution could be detected under UV-microscope, which is almost the same sensitivity as conventional ELISA. In addition, our assay system avoids dependence on complex equipment, such as flow cytometre in

Multiplexed Fluorescent Microsphere Immunoassay with which 0.02–0.6 ng/ml of antigens can be detected [17].

In conclusion, we have developed a single bead-based immuno-fluorescence assay to replace the conventional ELISA for the detection of snake venom. This rapid and simple method could reach the same sensitivity as conventional ELISA.

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