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Rapid and selective detection of experimental snake envenomation – Use of gold nanoparticle based lateral flow assay

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ABSTRACT

In this study, we have developed a gold nanoparticle based simple, rapid lateral flow assay (LFA) for detection of Indian Cobra venom (CV) and Russell's viper venom (RV). Presently, there is no rapid, reliable, and field diagnostic test available in India, where snake bite cases are rampant. Therefore, this test has an immense potential from the public health point of view. The test is based on the principle of the paper immunochromatography assay for detection of two snake venom species using polyvalent antisnake venom antibodies (ASVA) raised in equines and species-specific antibodies (SSAbs) against venoms raised in rabbits for conjugation and impregnation respectively. The developed, snake envenomation detection immunoassay (SEDIA) was rapid, selective, and sensitive to detect venom concentrations up to 0.1 ng/ml. The functionality of SEDIA strips was confirmed by experimental envenomation in mice and the results obtained were specific for the corresponding venom. The SEDIA has a potential to be a field diagnostic test to detect snake envenomation and assist in saving lives of snakebite victims.

1. Introduction

Snake bite is a fascinating, yet neglected public health problem resulting in life-threatening medical emergencies (WHO, 2010a,b; Kanchan et al., 2012; Kasturiratne et al., 2008). According to World Health Organization (WHO) estimates, around 35,000–50,000 people die due to snake bite each year in India. Worldwide about 3596 species of snakes were observed out of which 768 are venomous. In India, 52 amongst the 305 species of snakes are venomous, of which Indian cobra (*Naja naja naja*), Common krait (*Bungarus caeruleus*), Russell's viper (*Daboia russelii russelii*) and Saw-scaled viper (*Echis carinatus*) are responsible for the majority of snake bites and deaths in India and hence medically significant (Uetz, 1995; Whitekar, 2006; Brunda et al., 2006; Chippaux, 1998; WHO, 2010a,b; WHO, 2005). Fatalities and severe disabilities due to snakebite can be conquered with the rapid and judicial administration of the effective antidote, snake antivenom to only envenomed patients along with supportive treatments. During the administration of snake antivenom consideration is given to the possible associated anaphylactic reactions and its scarcity across the world (Simpson and Norris, 2009).

Treatment with antivenom along with specific supportive measures depends on whether venom is neurotoxic, hemotoxic or overlapping in nature. To aid specific supportive treatment identification of snake species is necessary as many of them mimic each other in appearance and color. In the case of snakebite, the average venom injected into the victim is variable, depending on the species, size, mechanical efficiency, and control of venom discharged by the snake (Hung et al., 2003). About 50% of bites by Malayan pit vipers and Russell's vipers, 30% of bites by Cobras and 5%–10% bites of Saw-scaled vipers do not result in any symptoms or signs of envenoming (Tun-Pe et al., 1991; Theakston et al., 2003). Considering these facts, the identification of snake species and envenomation determination has become crucial for the medical management of the snakebite victim.

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Snake bites are generally identified by the information of snake given by the patient, examining the bitten part, local signs and symptoms, biochemical analysis of urine and 20-min whole blood clotting test (20WBCT) (Warrell, 2010, 2012). These tests do not clearly indicate the snake envenomation severity and snake species. For decades, researchers are involved in developing a rapid, sensitive, simple, and reliable method for identification of species during snake envenomation. Many serological methods like immunodiffusion, immunoelectrophoresis, radioimmunoassay, and enzyme-linked immunosorbent assay (ELISA) are available to identify the species of snakes. Over the years, ELISA becomes the choice of test for detection of snakebites, since first described by Theakston et al. (1977) and thereafter extensively used for field applications and species-specific identification without reflecting any cross-reactivity within species (Selvanayagam and Gopalakrishnakone, 1999; Theakston, 1983).

Snake venoms composed of many proteins, organic and inorganic materials, and closely related species have many common proteins, which vary amongst individuals, environment, and season (Dong et al., 2003; Sharma et al., 2015). This complex nature of venom makes detection of specific venom more critical. Immunization with venom leads to the production of polyclonal ASVA having reactivity against most of the antigens injected, which exhibits cross-reactivity amongst the venoms (Selvanayagam et al., 1999; Gao et al., 2013). Use of these antibodies in the development of species diagnostic assays, may lead to ambiguity in the interpretation of results. Hence, the necessity to eliminate the cross-reactive antibodies, which can be achieved by passing immunoaffinity purified antibodies through venom bound affinity columns of cross reacting venoms to get SSAbs (Selvanayagam and Gopalakrishnakone, 1999; Gao et al., 2013). Although, SSAbs so obtained are used in various ELISA based assays and have a tremendous potential in the development of LFA for detection of snake venoms.

LFA is the simplest test and generally does not require any reagent for testing. The addition of samples like blood, saliva, urine or any liquid on the sample pad gives result within a few minutes and interpretation of the result does not require any instrumentation in many designs (Wong and Tse, 2008). The results obtained are mainly qualitative, in a Yes/No type and imparts an edge over ELISA without compromising sensitivity and specificity. LFA that emerged as a test of field application was first used for early detection of pregnancy using human urine sample. Today the application of LFA has expanded beyond clinical diagnostics to different areas like veterinary, agriculture, environmental health and safety, industrial testing, molecular diagnostics, forensic science, food industry, and more (Rong-Hwa et al., 2010; Sajid et al., 2014; Fang et al., 2011; Ju et al., 2010; Ching et al., 2012).

To address the problem of snake envenomation detection in our study, we have developed LFA for CV and RV detection on a single strip using SSAbs and anti-horse antibodies for impregnation on the nitrocellulose (NC) membrane at test and control lines respectively. Polyvalent horse ASVA conjugated with 25 nanometer (nm) gold nanoparticles was applied on the conjugated pad. On assembling the LFA components, we observed that LFA was sensitive, rapid, comparable, and reproducible for detection of experimental envenomation in Swiss albino mice.

2. Materials

Haffkine Biopharmaceutical Corporation Limited (HBPCL), Maharashtra, India, provided the venoms of Indian cobra (CV), Common krait (KV), Russell's viper (RV), and Saw scaled viper (EV) and ASVA. The venoms were distributed in small aliquots, lyophilized, and stored at −20 °C. Adult male Swiss albino mice (18–20 g) and New Zealand white male rabbits (2.0–3.0 kg) were maintained at HBPCL, Maharashtra, India and used for experimentation. Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) was purchased from DIFCO, USA and Bentonite powder was purchased from HiMedia, Maharashtra, India. Rabbit anti-horse IgG, goat anti-rabbit IgG - horseradish peroxidase (HRP) conjugates, Tetra methyl benzidine/Hydrogen peroxide (TMB/H_2O_2) substrate and Amicon centrifugal filters were purchased from Merck, India. Mabselect and CNBr-activated Sepharose - 4B were supplied by GE Healthcare Bio-Sciences Corp. USA. Nunc MaxiSorp 96 well flat bottom plates (Nunc, Denmark) were purchased from the manufacturer. Bovine serum albumin (BSA) was purchased from HiMedia, India. Gold Chloride ($AuCl_4$) 49% was purchased from Sigma, Bangalore, India. Oscar Medicare Pvt. Ltd. Delhi, India provided nitrocellulose (NC) membrane, conjugate pad, sample pad (mdi membranes, India), and cassettes. All other reagents and chemicals used were of analytical grade.

3. Methods

3.1. Protein estimation

Protein content was estimated by Bradford's method (1976), wherein equine immunoglobulin and BSA were used as standards for antibodies and snake venom protein estimations, respectively.

3.2. Snake venom LD_{50}

Snake venom LD_{50} was determined according to the method mentioned in WHO Guidelines for the production, control, and regulation of snake antivenom immunoglobulin's (2010). In brief, to determine LD_{50} of venom a group of six mice (18–20 g) were injected intravenously (iv) with 0.5 ml of graded venom dilutions (5.12 μg/ml to 12.5 μg/ml) assuming the nominal LD_{50} of venom in sterile normal saline solution. Deaths were recorded for 24 h and LD_{50} was estimated by Reed and Muench method (Saganuwan, 2011; Reed and Muench, 1938).

3.3. SDS -PAGE

SDS-PAGE of venoms was performed as protocol described by Laemmli (1970). Venoms were treated with Laemmli reducing sample buffer and resolved on 12% gel. Monovalent rabbit plasma and affinity purified antibodies were treated with native Laemmli sample buffer, resolved on 10% gel and silver staining was performed to visualize the bands.

3.4. Preparation of detoxified venoms

CV and RV were detoxified according to the procedure mentioned by Brunda et al. (2006). In brief, the detoxification of venom (1 mg/ml) was carried out by heating at 60 °C for 60 min (min) with immediately cooling in ice-cold water for 10 min, repeating the same process two times. Detoxified venoms were freshly prepared before each use.

3.5. Immunization of rabbits

Seroconversion to obtain antibodies against venom involves immunization with sub-lethal quantities, at periodic intervals, with or without adjuvants (Brunda et al., 2006; Waghmare et al., 2009). Briefly, New Zealand white male rabbits were selected for production of antibodies against CV and RV, respectively. The dose was prepared in an equal volume of normal saline (NS) and adjuvant, otherwise in NS and 0.5 ml injected subcutaneously (sc) on each