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Production and preclinical assessment of camelid immunoglobulins against *Echis sochureki* venom from desert of Rajasthan, India

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A B S T R A C T

Snakebite is a significant cause of death and disability in subsistent farming populations of rural India. Antivenom is the most effective treatment of envenoming and is manufactured from IgG of venom-immunised horses. Because of complex fiscal reasons, the production, testing and delivery of anti-venoms designed to treat envenoming by the most medically-important snakes in the region has been questioned time to time. In this study, we report successful immunisation of dromedaries (*Camelus dromedarius*) against the venom of Indian saw-scaled Viper- *Echis carinatus sochureki*. This study assessed the specificity and potential of camels immunised with venom of medically most important snake of Western India, the saw-scaled viper (*Echis c. sochureki*). Using WHO standard pre-clinical in vivo tests the neutralisation of the venom responsible for the lethal, haemorrhagic, coagulant and local necrotizing activities were measured, since these are the most significant effects that characterize envenoming by this species. The anti-venom was found significantly effective in the neutralisation of all these effects tested and thus, revealed further an immunological perspective, that camel IgG anti-venom (mono-specific) would be as efficacious as specific equine anti-venoms or even of better choice in treating snake specific envenoming.

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1. Introduction

Snake bite envenoming constitutes a significant public health problem with serious medical consequences that primarily affects rural farming community, particularly in tropical regions of Africa, Asia and Latin America (WHO, 2007; Warrell, 2010). It is a regional, environmental and an occupational bio-accident, rather, a ‘neglected Tropical disease’ as such. The Million Death Study concluded that in India snake bite causes about 45,900 annual deaths nationally (99% CI 40,900 to 50,900) (Mohapatra et al., 2011). Although antivenom is the most effective treatment for snakebite, they are frequently inaccessible to the millions of rural poor most at risk from snakebites (Harrison et al., 2009). By far the most surmountable challenges are those where one can deploy considerable experience; that is, in the design, production and assessment of new candidate antivenoms and in the refinement of current products to fulfil particular needs.

A key technical issue concerning the generation of new antidotes for snakebite envenoming is the design of improved immunisation mixtures in such a way that the resulting anti-venoms are effective against most venoms of the medically-relevant snake species within the geographical range where these antivenoms are intended to be used. This purpose is not trivial given the well-documented occurrence of venom variability at the genus, species, subspecies, population and individual levels (Chippaux et al., 1991). The variability of venom composition may endow snakes with the capability to adapt to different ecological niches. This is clearly evident for highly adaptable snake species of a wide geographical distribution in which allopatric venom variation may result in variable clinical presentations of envenoming (Calvete, 2013; Massey et al., 2012).

Echis c. sochureki is a highly medically important snake in desert of Rajasthan, India. It is capable of causing severe envenoming typified by incoagulable blood and potentially severe systemic bleeding in victim (Kochar et al., 2007). The Indian ‘polyvalent anti-snake venom serum’ is raised in horses using the venoms of the four most important venomous snakes (Indian cobra, *Naja naja*; Indian krait, *Bungarus caeruleus*; Russell's viper, *Daboia russelii*; saw-scaled viper, *Echis carinatus*). However, the validity of the

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concept of "the big four" is increasingly challenged by the discovery of other subspecies like *Echis c. sochureki* which is also important in desert regions in Rajasthan (WHO, 2010).

From the United States, a professional snake handler who suffered from exotic bite by *E. sochureki* has been reported and considered to be the single case of such bite. The clinical effects as evidenced as profound coagulopathy and gum bleeding with prolonged hypofibrinogenemia. Sixteen vials of Haffkine Indian polyvalent antivenom, was administered in this case with partial neutralisation of the *E. sochureki* venom (Weis et al., 1991) suggesting paraspecific action of horse immunoglobulin raised against *E. carinatus* venom for Indian polyvalent anti snake venom.

The dromedary is an important domesticated animal widely distributed over the north-western parts of India, playing important role in the social and economic life of the people (Khanna, 1990). From a logistical perspective camels represent an attractive alternative for antivenom production in this region. They are as facile to handle, immunize and bleed as horses; the yield of blood is equal to or more, that from a horse (Cook et al., 2010a). Besides, the unique physicochemical properties of camelid IgG offers intriguing possibilities to improve the clinical effectiveness of antivenom treatment (Harrison and Wernery, 2007). The antivenom presently in use in India has a record for frequently causing adverse reactions of either anaphylactoid or pyrogenic (Ariaratnam et al., 1999, 2001). These reactions occur in 60–80% of patients receiving anti venom (Ariaratnam et al., 1999; Kochar et al., 2007). There are significant barriers to use of anti venom in individual cases of snakebite in India, such as reluctance on the part of some physicians to use the product due to unfamiliarity or fear of adverse drug reactions (Simpson and Norris, 2007). Unfortunately these situations unduly increases "bite needle time" that is major determinant of snakebite outcome. Camel IgG is less immunogenic and less likely to activate the complement cascade than ovine or equine IgG (Herrera et al., 2005), suggesting that patients treated with Camelid IgG antivenom will suffer less from the anaphalactoid and serum sickness adverse effects. It is also possible that the unusual thermostability of camelid IgG (Omidfar et al., 2007) can be exploited to prepare antivenom that remains efficacious after room temperature storage in rural part of India the disease where belongs to.

As conspicuous from mentioned facts that, in this North West Thar desert region of India medically most significant snake is *E. sochureki,* against which specific antivenom is not component of Indian polyvalent anti venom and therefore a collaborative research work was under taken to raise the specific monovalent antivenom in dromedary camel against *E. sochureki* venom for preclinical assessment of camelid immunoglobulin.

2. Materials and methods

2.1. Animals

2.1.1. Snake and snake venom

E. sochureki of different age and sex were captured from desert of Bikaner District, Rajasthan after due permission from competent authority and maintained at due conditioned snake house in SP Medical College, Bikaner. After schedule milking, venom was frozen followed by lyophilisation and kept at - 20 °C for further use.

2.1.2. Dromedary camels

Disease free dully branded camels of different age and sex, maintained at National Research Center on Camel (NRCC), Bikaner, India. All the selected camels were under periodical health monitoring system by team of professionals from NRCC, Bikaner.

2.1.3. Mice

Swiss Albino mice under taken for the study were maintained in disease free conditions, provided with laboratory cubed diet and water *ad.lib*. The protocols used were approved by the Institutional Animal Ethics Committee for the Use of Laboratory Animals of the NRCC (Project VM-9).

2.2. Venom immunisation and sera collection

Two camels branded as M-42 and K-234 was immunised with venom of *E. sochureki.* Emulsified desired amount of venom antigen using Freund's complete adjuvant as per given protocol was used for primary immunisation followed by scheduled boosters with Freund's incomplete adjuvant. As per low dose, low volume, multisite approach (Sriprapat et al., 2003), emulsion was injected subcutaneously on both sides, near prescapular region of camels in area of highest lymphatic drainage. Pre immunisation blood was taken from the jugular vein of both the camels and sera were collected and stored at -20° C for further use.

After three boosters, sera of both animals were examined for neutralisation efficacy for coagulant effect of venom. It was observed efficacy of sera from M-42 was found significantly low as compared to K-234, so as two more camels K-236 and K-238 were enrolled for immunisation. The neutralisation potency of sera from both animals M-42 and K-236 were determined after following 16 and 13 booster respectively and found comparatively too low to continue. Therefore, both these animals were discontinued from the study. While monitoring, the antiserum from rest two camels revealed increasing trend of production of antibodies. After 25 boosters to K-234 and 22 boosters to K- 238 coagulant neutralisation efficacy (MCD-P) and mice protection assay (ED50) of both sera (pooled and separately) found at the desired level. Following that major bleeding was carried out and volume of 700 ml of blood was collected from jugular vein with all aseptic precautions, in a triple plastic bag with anticoagulant citrate phosphate dextrose solution in donor bag and saline adenine glucose mannitol solution in transfer bag (HLL Lifecare Limited, India) from each animals. Blood bags were centrifuged at 2100 rpm for 6 min at 22 °C with fast acceleration and de-acceleration. Plasma was transferred in plasma collecting bag and in donor bag preservative were added to blood cells, kept at 4 °C and after 48 h re-infused in respective camels.

2.3. Antivenom

Plasma from both animals were pooled and processed for purification of immunoglobulines by adjusting pH to 5.0 by using acetic acid followed by slow addition of 5% caprylic acid (Merck, Germany) with vigorous stirring for two hours at room temperature (Rojas et al., 1994). Supernant was separated by centrifugation at 8,000G for 30 Minutes, then diafiltered with sodium phosphate buffer (pH 7.2), and concentrated by Sterifile device fitted with 30 KD cellulose membrane (Millipore,USA) under Nitrogen gas pressure. Purified immunoglobulines passed through sterile 0.22 μm syringe filter and stored in 10 ml vials and kept at 4 °c. Protein concentration was measured using Folin's reagents (Lowry et al., 1951).

2.4. Preclinical assays

As per WHO guidelines, the range of LD50 for venom and ED50 for camelid anti-venom was determined by conducting assays to shorten the experimental time and minimizing number of experimental animals.

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