

The Development of Mucosal Immunization as an Alternative Approach for Production of Antisera Against Saw Scale Viper from Sindh

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Abstract. The reported cases of snake bite are 5.4 million per year, making the serious envenoming issue so, that WHO categorises cases of snake bite in neglected tropical diseases. Anti snake venom is produced by intramuscular injection of crude venom with different formulation of adjuvants to equine animals, but these intramuscular injections have several serious health impact in equine. Snake species categorised according to geographical locations, and there is a different composition of the snake venom at different locations. So, this research had been designed and evaluated new oral adjuvant formulation for viper family of Sindh, Pakistan. This is a animal immune based analysis consisting two Saw scale viper from Thar and costal area of Sindh because both have different habitat and morphological appearance. The venom toxin protein, combined with a mineral oil adjuvant for oral immunization. The adjuvant with venom *via* oral route produced of specific IgG and horse give maximum response with three doses of venom. The antibody titer was measured by ELISA showed significant results in both groups. For confirmation of specific antibodies isolated from horse serum, serum neutralization assay and immunodiffusion test were carried out. The snake venom with oral adjuvant immunization, is safe, efficient and time saving for production of equine Immunoglobulins IgG.

Keywords: oral immunization, venoms, saw-scale viper, immunoglobulins (IgG)

Introduction

The snake bite cases from Thar parker district of Pakistan are very distinct from other snake bite cases because of difference in snake variety. The saw-scaled vipers are the primary causes of snakebite morbidity and mortality in the desert area of Sindh, Pakistan. Similar circumstances are reported from African, Middle east countries, which creating problem in handling of snake bite cases Pouyani *et al.* (2016).

The worldwide estimated snake bite cases are 5.4 million out of which 2.5 million envenomation and 125,000 deaths per year. It is estimated that there are more than 1,000,000 snake bites in India alone leading to between 45000 to 50000 deaths per year Menno *et al.* (2017). South Asia has the highest rate of snake bite cases. India has highest mortality index; Pakistan has no accurate available data, but approximately 40,000 reported cases of snakebites and 8000 fatal cases per year are recorded by a literature search. In SriLanka, approximately 33,000 cases per annum, and in Nepal 1,000 death per year

recorded. The fatality rate is more or less same in the Asian region that is about 20% Parveen *et al.* (2017)

This serious envenoming problem and increase numbers of deaths has led WHO to recognise it as one of the neglected tropical diseases, chiefly accepted as an occupational hazard and a disease of poverty. So, the investigator started to design and achieve a better consideration of the compositional difference of venoms and assist the production of affordable, effective antivenoms against the majority of venomous snakes to solve the problem. The most effective treatment for snake envenomation is the administration of particular antivenom since many decades, which remains scarce in many parts of the world. Ratanabanangkoon *et al.* (2016)

Until launching a pharmaceutical product with complete identification, purity, protection and efficacy profiles anti-snake venoms development must follow the requirements of Good Manufacturing Practices (GMPs), including animal ethics. The identity of anti-snake venoms is determined by the equine animal species used as a source of immunoglobulin and the snake venoms source used as immunogens, León *et al.* (2018)

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Worldwide, many immunization methods have been discovered, such as recombinant toxins, synthetic peptides, or just the isolation of antigenic epitopes by different molecular digestion techniques, but in case of snake venom, the venomous animals essential to keep in captivity antivenom production open the era of new immunization approaches is considered relatively possible and adapted by manufacturers without introducing significant changes. Furthermore, since antivenoms gained by using novel immunization strategies have no differences as obtain from traditional antivenoms. The regulating bodies' endorsement processes should not restrain their product in the market. However, with these benefits, there is batch-to-batch difference is still expected, as a importance of immune response of immunized animals, Bermúdez *et al.* (2018)

In Pakistan, *Echis carinatus* (saw-scaled viper) are commonly found in desert areas, especially in agriculture land, cattle shades (Ther, Cholistan, and Makran in Baluchistan). It is a flat body with short tapered tail measuring about 0.4 to 0.6 meters long that travels rapidly in the opposite direction of the air flow. *Echis carinatus* have different variations of colours such as gray, olive or brown tone, back and some times both sides have different colours, with small white spots, (Parveen *et al.*, 2017; Odedara, 2017).

Saw-scale viper venom act as potent procoagulant toxins, which lead to venom-induced consumption coagulopathy (VICC) is the significant clinical manifestations in humans. Venom activates the clotting pathway by procoagulant toxins, resulting in clotting factor defect. The procoagulant toxin type varies between snakes species and can activate factor X and V, prothrombin activator or consume fibrinogen. (Rogalski *et al.*, 2017; Berling and Isbiter, 2015).

Therefore, the present study designed to establish a new strategy oral immunization in order to enhance the titer of antibodies against the venom of two saw-scale viper found in Sindh, Pakistan. As adjuvants are used for many decades for enhancement of immunogen activity, and in animals these adjuvants are used for production of hyperimmune sera, Valverde *et al.* (2017). Oil based vaccine are commonly practiced for veterinary controllable diseases which is designed for single dose for life long immune response against diseases studied by Park *et al.* (2016) The researchers have been started

working out the oral immunization technique, especially for the cure of *Mycobacterium tuberculosis*. So, we also design the mucosal immunization technique for production of specific anti snake venom against local viper family of Sindh, (Chambers *et al.*, 2017; Beltrán-Beck *et al.*, 2014).

Materials and Methods

Ethical considerations. All experimental horses were kept in the departmental of laboratory animal sciences Ojha campus, Dow University of Health sciences under the supervision of equine veterinarians. Ethical approval letter No (IRB-685/DUHS approval/2016/184) for the present study has been taken from Institutional Board Review Committee, Dow University of Health Sciences, Karachi.

Venom collection. Saw-scale viper have different groups which also have a different morphology and habitat. We collected venomous saw scale vipers from Ketti Bander, Kachall Border, Jhang, Qader Loo, Jatti, Shah Latife, Chill Mehar, (coastal areas) and Therparker district of Sindh (Fig. 1-2). The coastal area viper are still not categories as species or sub species of saw-scale viper its need another detail study but these snake have their on habitat. All snakes were kept in the snake house at the Laboratory Animal Science Department, DUHS.

Venom was collected manually with standard protocol provided by WHO. Then venom was centrifuged at 2500 RPM for 30 min. for cleaning the sample, freeze dried and stored at -20°C till used.



Fig. 1. Saw-scale viper of coastal area (Sindh).



Fig. 2. Saw-scale viper of Tharparker (Sindh).

Animal study plan. In breed line NIMRI mice strain weighting from 20–30 g were selected for venom dose calculation (lethality testing and neutralization Assays), and Local horses age ranges from 3 to 8 years, weight 200–300 Kg was included in this study for immunization protocol, purchase from local farmers after certification by a veterinarian.

All experimental groups (large and small animals) were kept in Laboratory Animal Science Department, DUHS under supervision of veterinary consultant.

Venom protein estimation. The venom protein was estimated by standard Bradford method. The basic principle of this assay is the conversion of Coomassie Brilliant Blue G-250 dye into different colours that is neutral (green), anionic (blue) and cationic (red). The existence of protein in the dye forms a strong, non-covalent complex with the protein's carboxyl group by Van der Waals forces and amino group through electrostatic interactions, which is measured by the use of a calorimetric absorbance reading. Cheng *et al.* (2016) (Bradford kit provided by Thermo Scientific™)

- Tharparker saw-scale viper protein calculated as 90 μ g/ μ L of venom.

- Coastal saw-scale viper protein calculated as 120 μ g/ μ L of venom.

The venom protein was calculated for LD¹⁰⁰ in mice. Hosseini *et al.* (2017)

Toxicological studies. Toxicological study was done in two groups of NIMRI mice. Each dilution tested on five mice with one control. One group for coastal saw-scale viper and 2nd group for Tharparker district saw-scale viper (SSV) which are shown in Table 1.

The venom was serially diluted by 1:9 ratio and from each dilution injected to five mice by intraperitoneal route. Dead or alive mice were observed and recorded from 24 to 48 h, respectively. WHO guidelines (2010).

Animal husbandry conditions. Good animal handling, high nutritional diet, water and biological stress free conditions were provided. Daily veterinary examination and treatment have been provided as per requirement.

Preparations of adjuvants. The small animals NIMRI mice was used to find out the ideal concentration of (adjuvants with venom protein combinations) to develop an oral adjuvant formulation by (v/v) Tween²⁰ Tween⁸⁰, Mineral oil, PBS and saw-scale snake venom. These were mixed with a vortex mixture till dense creamy white or light brown colour (emulsion) appeared, Shah *et al.* (2015). The orally by 0.5 mL from each ratio given feeding tube in five mice for tolerability, shown in Table 2.

Protocol for monovalent immunization of horses. Ten horses were allotted for research trial as given a unique number, name and after passes the quarantine time, horses distributed in two groups each group, have five in for protocol 1 and protocol No. 2 (Thar SSV and costal area SSV respectively). Prior to immunization of horses, serum was collected from all animals and stored as negative controls, shown in Fig. 1.

Isolation and testing of horse I_gG. We use caprylic acid for precipitation technique for commercial production of anti-snake venom. Quantification of horse

Table 1. Toxicological studies into two groups.

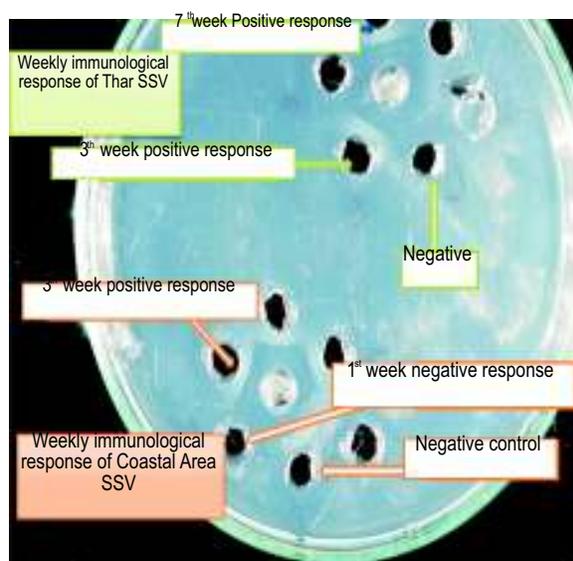
Snake venom	Dilution 1 0.5mL/mice	Dilution 2 0.5mL/mice	Dilution 3 0.5mL/mice	Route of injection 0.5mL/mice	Control
Costal SSV	0.5	0.5	0.5	Intraperitoneal	0.1
Thar SSV	0.5	0.5	0.5	Intraperitoneal	0.1

Table 2. Preparation of venom protein formulation

Snake Venom fixed volume	Adjuvant ratio1 10% (v/v) 0.5 mL/mice	Adjuvant ratio 2 20% (v/v) 0.5 mL/mice	Adjuvant ratio 3 30% (v/v) 0.5 mL/mice	Route of injection	Control
Costal SSV	05	05	05	Orally	01
Thar SSV	05	05	05	Orally	01

IgG against snake venoms in the different groups of horses sera was analyzed by modifying enzyme link immuno sorbent assay (ELISA), the absorbance values read at 492 nm with the help of ELISA reader, and the results were recorded.

The specific immunoglobulin against snake venoms, neutralised by agar gel immunodiffusion test was performed using an Ouchterlony double immunodiffusion technique which is shown in Fig. 3.

**Fig. 3.** Immunodiffusion results of Thar and coastal area saw-scale viper.

Statistical analysis. All data were calculated and analysed by SPSS version 16 with paired sample T test for standard deviation.

Results and Discussion

LD¹⁰⁰ for snake venom. In mouse strains, the mortality of saw-scale viper snake venom was checked. It was found that intraperitoneal route was ideal in NIMRI mice for calculation of LD¹⁰⁰. The result of LD¹⁰⁰ of costal area SSV venom was 480 µg /25 g body weight of mice and for Thar SSV venom was 360 µg/25 g.

Table 4 and Fig. 4 shows the antibodies (IgG) titer measured by ELISA. The result was statistically

Table 4. Comparison of antivenom of Thar SSV with costal area SSV produced by oral adjuvant measured by ELISA

Weeks	Antivenom of Thar SSV (µg/µL of IgG)	Antivenom of costal area SSV (µg/µL of IgG)
0 week (Pre-venom) bleed	0.0	0.0
1 week after the first dose	0.6	0.8
3 weeks after Second dose	1.7	1.9
7 weeks after 3 rd dose	2.9	3.1

*Elisa (absorbance at 492 nm); Statistically significant differences between oral adjuvant (P < 0.058).

Table 3. Protocol for monovalent immunization of Horses

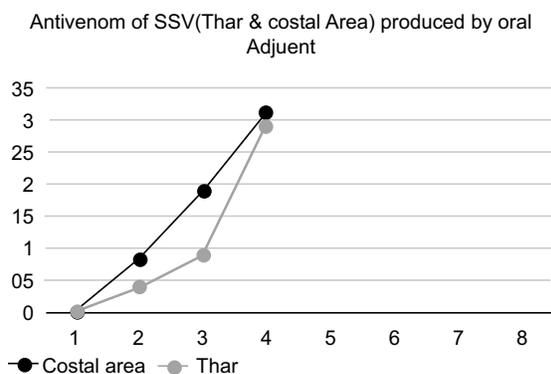
Immunization week	Adjuvant composition	Protocol 1 Thar SSV (venom)	Protocol 2 costal area SSV (venom)	Route
1	Tween ²⁰ & Tween ⁸⁰ Mineral oil, PBS	500 µL	500 µL	Orally
3	Tween ²⁰ & Tween ⁸⁰ Mineral oils, PBS	250 µL	250 µL	Orally
7	Tween ²⁰ & Tween ⁸⁰ Mineral oils, PBS	200 µL	200 µL	Orally

Table 5. Serum neutralization assay of Thar SSV venom by *in vivo* studies

Weeks	Fixed amount of LD ¹⁰⁰ venom (μL)	I _g G Antibodies (μL)	Vivo studies	Live mice (Mice)	Dead mice	Control mice one for each group	Remarks
0 Week (Prevenom)	40	150	5	----	5	Alive	
After 1 st week	40	150	5	----	5	Alive	
3 rd Week	40	150	5	4	1	Alive	80%
7 th Week	40	150	5	5	0	Alive	100%

Table 6. Serum neutralization assay of costal area SSV venom by *in vivo* studies

Weeks	Fixed amount of LD ¹⁰⁰ venom (μL)	I _g G Antibodies (μL)	Vivo studies (Mice)	Live mice	Dead mice	Control mice one for each group	Remarks
0 Week (Prevenom)	40	150	5	----	5	Alive	
After 1 st week	40	150	5	----	5	Alive	
3 rd Week	40	150	5	4	1	Alive	80%
7 th Week	40	150	5	5	0	Alive	100%

**Fig. 4.** Comparison of antivenom of Thar SSV with costal area SSV produced by oral adjuvant measured by ELISA.

significant (P value 0.058) means both the groups of SSV responds very well with mucosal immunization technique given by oral route.

Table 5 and 6 showed serum neutralization assay *in vivo* studies. Both groups of SSV venom produced sufficient antibodies when gave as oral adjuvant formulation and provided 100% protection after three doses of immunization.

Conclusion

There are no reference articles worldwide for use of anti-snake venom preparation, so the present study was

carried out to develop an oral adjuvant formulation for delivery of snake venom to mucosal surface which have well defined lymphoid system for production of Anti snake venom immunoglobulin which also have the same potential as by the injectable path.

The majority of licensed vaccines was administered designed for subcutaneous or intramuscular injection for activation of systemic humoral immunity (*e.g.* antibody production) against specific antigen, with partial cellular immunity (*e.g.* T cell-mediated) and at mucosal surfaces only produce poor protection. Studied by Ramirez *et al.* (2017)

In contrast, in oral immunization the pathogen or toxin direct interact with mucosal surfaces induces successfully mucosal antibodies (I_gA) and cell-mediated immune responses which ultimately activate systemic antibody response (I_gG), Newsted *et al.* (2015)

Since, many decade several adjuvants have been used for several decades to develop stronger immune response with least adverse effects. These adjuvants induce better response to immune system than other formulation. Our findings strongly support the efficacy of oral adjuvant-carrier system for the effective induction of a specific immune response and targeted delivery system for production of anti-venom immunoglobulin described by Mehrabi *et al.* (2018)

More than 70 years adjuvants are use to design vaccines to improve the immune response to particular antigens,

increase the duration of antibody response, and decrease the frequency of vaccination doses and the amount of antigen in each dose. Although water-in-oil emulsions are used for the long-term production of humoral immune response in poultry vaccines, as they are not suitable to be used in human vaccines, because they induce chronic inflammatory response at the injection site studied by Jafari *et al.* (2017) So, we used oil emulsion for oral route vaccination in equine animals Meenatchisundaram *et al.* (2008) showed in his research the maximum titer of immunoglobulin I_gG against saw-scale viper concentration varied in the range of 0.3–6.7 mg/mL of I_gG for Russell's viper venom, and 0.5–6.9 mg/mL of I_gG for Saw-scaled viper venom throughout the immunization period. This study is comparable with our study which shows Immunoglobulin G titer is 0.6–2.9 ug/uL for Thar saw-scale viper and 0.8–3.1ug/uL for costal area saw-scale viper.

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Conflict of Interest. The authors declare no conflict of interest.

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