



IMMUNOLOGICAL AND PHYSIOLOGICAL RESPONSES IN HORSES AND CAMELS AGAINST PAKISTANI VIPER VENOM IMMUNIZATION FOR SNAKE ANTIVENOM IMMUNOGLOBULIN PRODUCTION

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ABSTRACT

The treatment for snakebite victims is antivenom immunoglobulins. Modern immunization methods for horses and camels with Pakistani viper venoms are rare, despite maintaining animal safety and efficacy. The study aimed to assess the horses' and camels' immunoglobulins production response against Pakistani viper snake venoms. Throughout immunization, each horse (N=3) and camel (N=3) was immunized with *Echis carinatus sochureki*, *Daboia russelii*, and *Echis carinatus multisquamatus* venoms using Freund's complete or incomplete adjuvants following previously reported low-dose multi-site immunization method. The study compared the Lumps size, body weight, total protein, albumin, and globulin concentration every 15 days, recorded body temperature for three days after each immunization dose, and confirmed immunoglobulin G production through double immune diffusion gel diffusion and SDS-PAGE electrophoresis. The study found that Freund's complete and incomplete adjuvant induced reactions (lumps) were more significant in camels than in horses ($p < 0.05$). No significant weight gain or loss was noted ($p > 0.05$). After immunization, the increase in body temperature was temporary, and horses were more stable than camels. Total protein and globulin concentrations increased after the 3rd and 4th immunization doses, while albumin concentrations decreased in horses and camels significantly ($p < 0.05$) compared to their basal concentrations. The antigen-antibody precipitin line in double immune diffusion gel diffusion and IgG bands in SDS-

PAGE gel confirmed the development of snake antivenom immunoglobulins against viper venoms. In conclusion, horses and camels showed positive response to producing antivenom immunoglobulins against Pakistani viper venoms, but camels experienced severe physical impact compared to horses.

Keywords: Immunization, Antivenom, Immunoglobulins G, *Echis carinatus sochureki*, *Daboia russelii*, *Echis carinatus multisquamatus*, Viper snakes, Pakistan

Introduction

Snakebite envenoming is a significant health concern that has been widely recognized and neglected worldwide, especially in emerging countries of Asia and Africa.(1, 2) Recent estimates that snake bites are 1.8 - 2.7 million yearly, with 81,000 to 138,000 mortalities.(3) India has the most significant rate of snakebites, with 80,000 incidents and 11,000–46,000 fatalities recorded yearly.(4) It is difficult to obtain accurate data on snakebite cases in Pakistan, but it is estimated that there are at least 40,000 incidents per year, resulting in 1000–8200 deaths.(5) In Pakistan, the frequency of viper snake bite cases is more than elapid, and Sindh is the most affected province of Pakistan. The World Health Organization classifies Pakistan's Saw-scaled Vipers (*Echis carinatus* species) in category 1, which is highly venomous.(6)

Antivenom immunoglobulins have been developed using animal species like horses, sheep, goats, and camels. Horses are commonly utilized globally in producing antivenom immunoglobulins due to their ease of handling, ability to adapt to various climates, and high serum/plasma production. Immunization and immunoglobulin purification methods are well-established(7-9), whereas camels have been used only for experimental purposes.(10, 11)

Practically, animal immunizations should generate long-lasting, high-titer antibody responses to the lethal components of the venom (s). Various vaccination techniques have been used to create immunoglobulins against snake venoms in large animals, with the help of numerous adjuvants.(12-14) Therefore, the resulting antibody titer can be varied based on the selection of animals, adjuvants, and immunization protocols. When immunized with deadly snake venoms and adjuvants, animals develop multiple local reactions (such as tissue necrosis, edema, abscess, large nodule formation, severe inflammation, fistula, and fibrosis) at the injection site.(15, 16) Physical and biochemical factors were recorded in many studies.(7, 15, 17)

Antivenom presently developed in Pakistan merely using horses, which are well adapted to the arid climate of much of Pakistan. However, it is expensive to maintain in Pakistan, leading to an increased cost of antivenom immunoglobulins production. On the other hand, camels are also well adapted to the dry conditions in Pakistan and cost-effective to maintain, which may offer a cheaper option for antivenom immunoglobulin production. Most importantly, camelid IgG proved to be more effective(10), less immunogenic(18) and has better thermal stability(19) than equine IgG. These unique features of camel immunoglobulins make them more appealing to Pakistan, particularly in remote areas of Sindh, where there is no provision for cold storage maintenance and other health facilities.

The study aimed to evaluate the immune response of horses and camels in producing immunoglobulins against three viper snake venoms. Additionally, the study aimed to analyze and compare the impact of the venoms/adjuvants on various physical and biochemical alterations.

Materials and Methods

The research was piloted in compliance with the rules and code of practice regarding the use of animals in scientific studies, as mandated by the Institutional Bioethical Committee, University of Sindh, Jamshoro. An institutional and Bioethical committee approved the study protocol via letter Ref No. IOB./78/2023, dated 15/02/2023 prior to carry the present study.

Anti-Snake Venom Serology Laboratory, Sakrand Shaheed Benazirabad gifted the dried viper venoms of *Echis carinatus sochureki* (ECS), *Daboia russelii* (DR), and *Echis carinatus multisquamatus* (ECM)) native to Sindh. Freund's complete and incomplete adjuvants were acquired from Sigma-Aldrich, USA, while the total protein and albumin reagent kit was obtained from Merck,

Germany. Moreover, analytical-grade chemicals and reagents were purchased from authorized distributors.

Selection and Immunization of horses and camels

Three male horses aged 3-4 years, weighing 387-406 kg, and three male camels aged 4-5 years, weighing 490-535 kg, were selected for present study. The animals received additional food fortified with minerals and vitamins throughout the immunization regimen.

Each horse and each camel was immunized with three viper venoms by adopting a low-dose multi-site protocol.(7, 10, 20) Freund’s complete and incomplete adjuvants were used for the first and second immunization doses, respectively, whereas, for subsequent boosters, saline was used. To prepare the immunogen cocktails, the desired working concentration of individual venoms was dissolved in sterile saline and mixed with adjuvants in a 1:1 ratio using sterilized syringes connected with a 3-way stopcock.(6) The horses and camels received immunogen doses (02 ml total for each venom) subcutaneously into their necks at 30 sites (10 sites/venom) in a volume of 0.2 ml per site. The concentration of venoms was increased by gradually after every dose in both horses and camels. All animals received venom injections throughout 60 days at around 15-day intervals. Before each immunogen dosage, blood samples were collected and centrifuged for serum separation and kept at -20 °C for further investigations. All animals of both species experienced the same protocol except for a slight change in venoms concentration for camels. Details are given in Table 1.

Table 1: Details on the immunization scheme of horses and camels.

Immunization #	Intervals (Days)	Venom (mg) / Dose			Dose Composition (1:1 v/v)	Dose Volume / Animal	Volume / Site; Number of sites	Total Venom, Volume, and Sites / Animal	
		Venom (s)	Horses (N=3)	Camels (N=3)				Horse	Camel
1 st	0	ECS	1	2	ECS + FCA	02 ml	200 µl; 10 sites	03 mg; 06 ml; 30 Sites.	06 mg; 06 ml; 30 Sites.
		DR	1	2	DR + FCA	02 ml	200 µl; 10 sites		
		ECM	1	2	ECM + FCA	02 ml	200 µl; 10 sites		
2 nd	15	ECS	2	4	ECS + FIA	02 ml	200 µl; 0 sites	06 mg; 06 ml; 30 Sites.	12 mg; 06ml; 30Sites.
		DR	2	4	DR + FIA	02 ml	200 µl; 10 sites		
		ECM	2	4	ECM + FIA	02 ml	200 µl; 10 sites		
3 rd	30	ECS	6	8	ECS + NS	02 ml	200 µl; 0 sites	18 mg; 03 ml; 15 Sites.	24 mg; 03 ml; 15 Sites.
		DR	6	8	DR + NS	02 ml	200 µl; 10 sites		
		ECM	6	8	ECM + NS	02 ml	200 µl; 10 sites		
4 th	45	ECS	8	10	ECS + NS	01 ml	200 µl; 0 sites	24 mg; 03ml; 15Site s.	30 mg; 03 ml; 15 Sites.
		DR	8	10	DR + NS	01 ml	200 µl; 10 sites		
		ECM	8	10	ECM + NS	01 ml	200 µl; 10 sites		
5 th	60	ECS	10	15	ECS + NS	01 ml	200 µl; 0 sites	30 mg; 03ml;	45 mg; 03 ml;

	DR	10	15	DR + NS	01 ml	200 µl;10 sites	15Site s.	15 Sites.
	ECM	10	15	ECM + NS	01 ml	200 µl;10 sites		

Abbreviations: **ECS**, *Echis carinatus sochureki*; **DR**, *Daboia russelii*; **ECM**, *Echis carinatus multisquamatus*; **FCA**, Freund's Complete Adjuvant; **FIA**, Freund's Incomplete Adjuvant.

The post-immunization reactions caused by viper venoms mixed with adjuvants among horses and camels were compared regarding local reaction (lumps), body weight and temperature during immunization. The local reactions (lumps) diameters were measured in centimeters (cm) to assess their severity.(7, 21) These measurements were taken every 15 days till complication of 60 days. The body temperature was evaluated in °F and compared for three consecutive days following each immunization using an infrared digital thermometer. The body weight was recorded (in Kg) and compared after 15 days following each immunization using a digital weight platform scale. The basal body temperature and weight were recorded for all animals before the start of the immunization program.

The blood specimens were collected from the jugular vein of horses and camels using sterile clot activator test tubes with gel separators before each immunization dose and left at 20 –25 °C for serum separation. Biochemical parameters, including total protein, albumin, and globulin concentration (g/dl), were measured using a clinical-grade chemistry analyzer (Micro Lab 300, Merck Private Ltd, Germany). The quantification of total protein was conducted with the biuret kit technique(22), while the level of albumin was measured using the Bromocresol Green (BCG) colorimetric technique.(23) For Globulin values, the concentration of albumin was deducted from the concentration of total protein.(22)

The Antigen-Antibody interaction of hyperimmunized serum (from horses and camels) against three viper venoms (ECS, DR, and ECM) was performed using a 1% agarose gel in the immune diffusion test. The whole procedure was performed by a reported method.(24) Whereas, the SDS-PAGE Gel Electrophoresis of hyperimmunized crude serum (horses and camels) was done by the Laemmli method.(25)

Results:

The reaction size (lumps) due to viper venoms mixed with Freund's complete adjuvant gradually increased in horses and camels. The camels had significantly larger lump sizes compared to horses after each immunization dose ($p < 0.05$). However, reactions (lumps) in horses decreased after 45 days, while camels had even more increased reaction sizes at this stage, as shown in Table 2. In contrast, the reaction size (lumps) due to viper venoms mixed with Freund's incomplete adjuvant increased significantly ($p < 0.05$) at all-time intervals throughout the immunization process. However, the reaction size (lumps) after 30 and 60 days were found to be highly significant ($p < 0.001$), as shown in Table 2. The study found that the reaction size (Lumps) due to viper venoms mixed with Freund's incomplete adjuvant showed significantly larger reaction sizes (lumps) than Freund's complete adjuvant in all immunization points in both horses and camels, however camels had significantly larger lumps sizes than horses in both Freund's complete and incomplete adjuvants.

Table 2: Comparison of reaction size (lumps) due to viper venoms mixed with Freund's complete/incomplete (FCA/FIA) adjuvant among horses and camels.

Intervals	Adjuvant	Horses	Camels	<i>p-Value</i>
		Mean ± SD (cm)	Mean ± SD (cm)	
15 Days	FCA	2.26 ± 0.71	2.62 ± 0.73	0.001
	FIA	2.51 ± 0.68	2.83 ± 0.70	0.002
30 Days	FCA	3.52 ± 0.73	3.88 ± 0.73	0.001

	FIA	3.81 ± 0.72	4.26 ± 0.74	< 0.001
45 Days	FCA	4.48 ± 0.75	4.82 ± 0.86	0.005
	FIA	4.89 ± 0.94	5.29 ± 0.79	0.002
60 Days	FCA	3.66 ± 0.73	5.71 ± 1.05	< 0.001
	FIA	4.08 ± 0.87	6.12 ± 0.83	< 0.001

Horses and camels experienced increased body temperature compared to their initial values when exposed to venom/adjuvants. There was a significant rise in body temperature ($p < 0.05$) in horses during the first day of 1st, 2nd, and 3rd immunization doses and on the second and third days of 1st and 2nd immunization doses. Similarly, camels showed significant changes in body temperature ($p < 0.05$) during the first day of the 2nd to 4th immunization doses and on the second and third days of the 1st to 3rd immunizations. However, the body temperature of horses and camels were slightly stabilized by the 4th and 5th immunization, respectively. It indicated that the effect of venom/adjuvants on basal body temperature was temporary and did not persist after repeated immunizations in both species, as shown in Table 3.

Despite the changes in the weight of all animals throughout the immunization regimen, a slight increase in the weight of horses and camels compared to their initial values were recorded as insignificant ($p > 0.05$), as shown in Table 4.

Table 3: Comparison of body temperature between horses and camels with their basal body temperature after each immunization dose.

Immunizations	Day 1		Day 2		Day 3	
	Temperature of Horses (°F)	Temperature of Camels (°F)	Temperature of Horses (°F)	Temperature of Camels (°F)	Temperature of Horses (°F)	Temperature of Camels (°F)
1st	101.50 ± 0.50	105.47 ± 0.76	102.03 ± 0.40	106.60 ± 0.66	102.67 ± 0.76	107.67 ± 0.29
<i>p</i> -Value	0.032	0.183	0.010	0.019	0.008	0.002
2nd	101.50 ± 0.50	106.07 ± 0.31	102.57 ± 0.49	106.83 ± 0.29	103.17 ± 0.58	108.13 ± 0.42
<i>p</i> -Value	0.032	0.030	0.020	0.007	0.003	0.001
3rd	101.67 ± 0.76	106.17 ± 0.58	101.17 ± 0.85	106.87 ± 0.51	100.53 ± 0.85	106.43 ± 0.51
<i>p</i> -Value	0.037	0.037	0.102	0.009	0.304	0.020
4th	101.13 ± 0.81	105.93 ± 0.40	100.7 ± 0.85	105.83 ± 0.76	100.27 ± 0.75	105.23 ± 0.64
<i>p</i> -Value	0.103	0.046	0.233	0.092	0.440	0.263
5th	100.77 ± 0.75	105.53 ± 1.00	100.23 ± 0.68	105.10 ± 0.96	100.17 ± 0.76	104.67 ± 0.58
<i>p</i> -Value	0.193	0.195	0.458	0.363	0.500	0.389

Note: The basal body temperature of horses and camels were 100.17 ± 0.76 and 104.83 ± 0.76 °F, respectively. Measurements were taken for consecutive three days following each immunization. *P*-Value of each day calculated from their respective basal temperature.

Table 4: Comparison of weight (Kg) between basal weight and after each immunization doses in horses and camels.

Immunizations	Weight of Horses Mean ± SD (Kg)	<i>p</i> -Value < 0.05	Weight of Camels Mean ± SD (Kg)	<i>p</i> -Value < 0.05
1st	394.9 ± 9.49	0.474	512.57 ± 21.36	0.467
2nd	394.67 ± 8.22	0.461	513.6 ± 21.18	0.489

3rd	393.4 ± 8.34	0.397	512.47 ± 21.31	0.465
4th	396.37 ± 8.90	0.454	514.17 ± 20.31	0.499
5th	397.5 ± 9.18	0.400	515.67 ± 19.22	0.467

Note: Measurements were taken fifteens days after each immunization. The basal weight of horses and camels were 395.43 ± 9.52 and 514.13 ± 22.68 kg, respectively. *P*-values were calculated from their basal weight.

In horses, the total protein concentration was significant increased ($p < 0.05$) after 3rd, 4th, and 5th immunizations, while camels had substantial values ($p < 0.05$) after their 4th and 5th immunizations. The albumin concentration ($p < 0.05$) decreased significantly in horses after the 3rd, 4th, and 5th immunization doses and in camels after the 4th and 5th immunization doses. Likewise, total protein and globulin concentrations significantly ($p < 0.05$) increased in horses after the 3rd, 4th, and 5th immunization doses, while camels experienced the same trends after their 4th and 5th immunization doses, as shown in Figure 1.

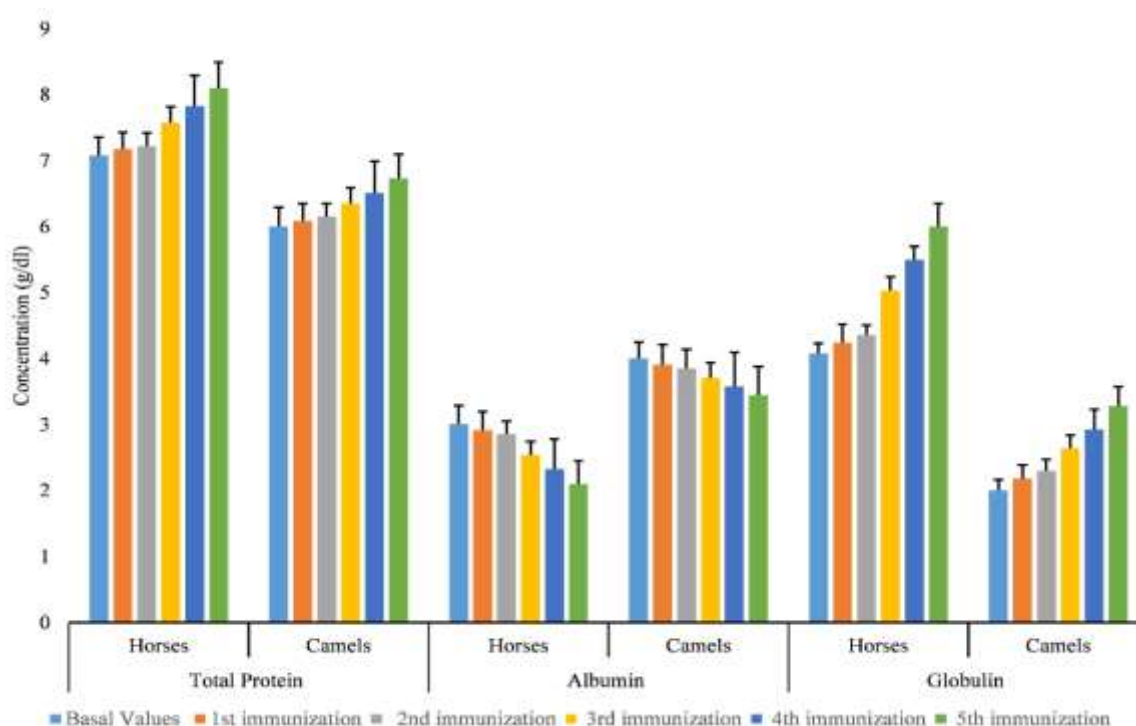


Figure-1: Comparing the effects of venom/adjuvants on various biochemical parameters in horses and camels throughout immunization.

Note: values are stated in average. When measuring total protein levels in horses, the standard deviation range is between 0.21 and 0.29 whereas in camels, this range is between 0.21 and 0.30. As for albumin levels, the standard deviation in horses is between 0.15 and 0.29, whereas in camels, it falls between 0.20 and 0.25. Finally, when it comes to globulin levels, the standard deviation range for horses is between 0.20 and 0.51, while in camels, it varies between 0.29 and 0.43.

The double immune diffusion gel images demonstrated the clear and prominent precipitation lines when specific antigens (venoms) were allowed to diffuse with the developed antibodies in the hyperimmunized crude serum of camel (Figure 2A) and horse (Figure 2B).

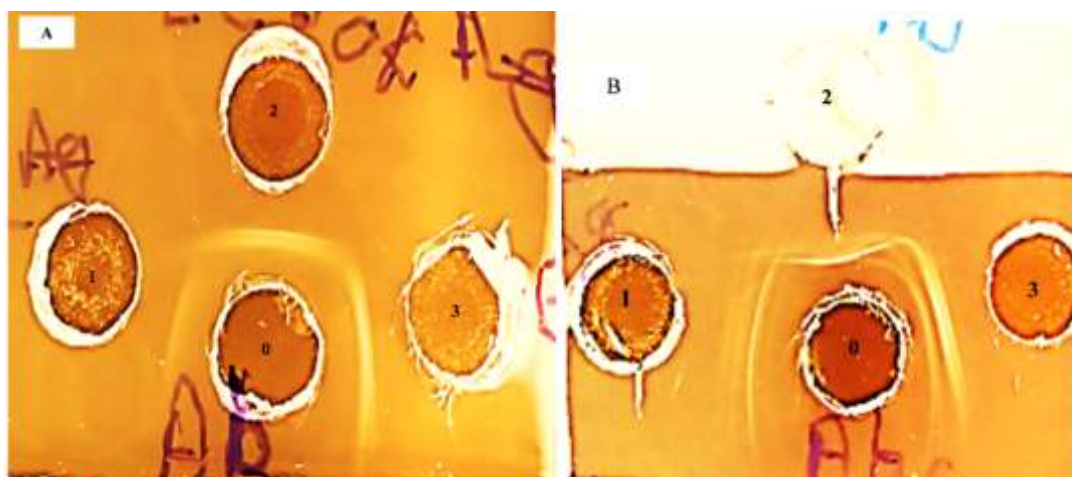


Figure 2(A & B): Immunodiffusion of viper venoms (ECS, DR, ECM) and hyperimmunized camel (**Figure 2(A)**) and horse (**Figure 3(B)**) serum.

Wells 0-3: 0 for Camel and horse hyperimmunized serum, 1 for *Daboia russelii* DR Venom, 2 for *Echis carinatus sochureki* ECS Venom, 3 for *Echis carinatus multisquamatus* ECM Venom.

The SDS-PAGE gel revealed a mixture of proteins with varying molecular weights, including bands of immunoglobulin G (IgGs) that were visible as dense bands spread across a wide area in the gel. In non-reducing conditions, horse crude plasma (HCP) lane indicated a protein band of IgG \approx 160 kDa, while that of Camel crude plasma (CCP) lane showed two protein bands of IgG \approx 160 & 100 kDa as shown in Figure 3(A), which dissociated into smaller proteins and migrated downward in the reducing gel as indicated in Figure 3(B).

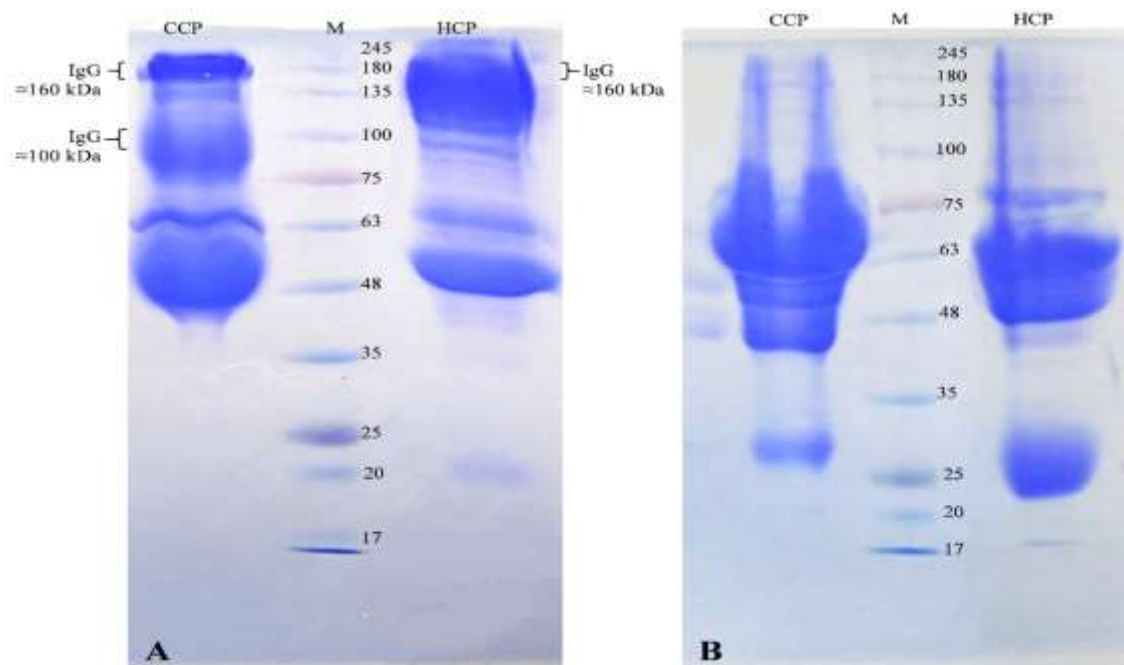


Figure-3(A&B): SDS-PAGE electrophoresis (10%) analysis of hyperimmunized crude plasma of horses and camels under non-reducing (**Figure 3(A)**) and reducing conditions (**Figure 3(B)**).

HCP, Horse crude plasma; **CCP,** Camel crude plasma; **M,** protein marker; **kDa,** Kilo Dalton.

Discussion

Antivenom immunoglobulins are the only remedy to treat snake envenomings in the world, and currently, immunoglobulins are produced from the animal's blood. Horses have been the preferred animal for the manufacturing of antivenom for generations. However, there have been experimental evaluations of camelids' capacity to produce immunoglobulins targeting the venoms of Viperidae snakes in West Africa and South America.(10, 11, 26)

Adjuvant implementation in immunization is a widely accepted and practical approach for producing snake antivenom immunoglobulins.(27) The primary aim of using adjuvants is to activate the animal's immune system for optimal production of immunoglobulins in response to venom(s). The injection of venoms/adjuvants can potentially induce localized edema, abscess formation, fistula development, and fibrotic changes at the injection site.(7, 28)

The existing research findings indicate that the adopted immunization method has shown to be very effective in producing immunoglobulins against immunized viper venoms. The study (Table 2) found that Freund's Complete and Incomplete adjuvant in horses and camels resulted in varying degrees of local inflammatory reactions, as reported in horses.(15) Moreover, horses experienced worse itching in existing research, as reported(8) , although they had minimal reaction sizes (lumps) compared to camels as shown in Table 2. Camels experienced the development of numerous giant lumps that persisted for over eight to ten months; over time, the size of these lumps increased from initial and turned brown without any fluid discharge, except a few; comparable results were documented in alpacas.(16) Subsequently, the horse's lumps progressed into abscesses which ultimately ruptured, leading to wound healing, and approximately 15-18 weeks later, these nodules exhibited little presence of tiny lesions; these observations were broadly similar to that observed previously.(7, 17) This quick recovery in horses may result from extensive care or the implementation of local remedies. Camels with such severe skin reactions to Freund's complete or incomplete adjuvants can be problematic during the next round of immunization and may need to withdraw from the herd. Administering Freund's complete or incomplete adjuvants injections to camels is difficult due to their thick skin, especially in the neck. The lumps in their skin were hard to puncture, making it challenging to give multiple injections. To avoid complications in camels during immunization in camels, this research suggests evaluating different dosing sites, such as the armpit or groin areas, as these areas are usually adopted in sheep(29) and instead of Freund's complete or incomplete adjuvants, start immunization with water-based adjuvants such as GERBU adjuvant(10) or the montanide family of adjuvants, including IMS 3012, ISA 206, and ISA 35.(17) These recommendations may help to minimize potential issues in future studies.

All camels and horses developed a fever for a few days after each immunization except the last two doses as shown in Table 3; this indicates that the effect of venom/adjuvants on basal body temperature was temporary and did not persist after repeated immunizations in both species. The rise in body temperature was previously reported.(7) Contrary to previously reported findings(17) , elevated body temperatures influenced the animals' food intake, but the animals' behavior returned to normal shortly after the temperature dropped. Additionally, loose stools were observed in camels during immunization. Statistically, no substantial increase or decrease in weight was observed in all animals of both groups as shown in Table 4; similar results were reported previously.(7)

The importance of assessing the health of animals during immunization using biochemical testing is due to the minimal information on adjuvants used in the production of snake venom antibodies in Pakistan. The elevation in total protein concentration during immunization could result in increased globulin concentration. In addition to gamma globulins, specific acute-phase proteins such as serum amyloid A, alpha-macroglobulin, and hepato-globulins also rise in acute-phase conditions, contributing to a slight rise in total protein levels as reported by Husby *et.al.* 1992.(30) Additionally, when venom and an adjuvant are used together, it can cause antigens and antibodies to interact and form immunological complexes(31) , may be responsible for the increased total protein levels in camels and horses (Figure 1). After immunization doses, there may be a connection between lower albumin levels and sudden cytokine-induced responses. Cytokines influence the liver and cause excess production of proteins such as amyloid A and fibrinogen, which leads to a decline in albumin

synthesis.(32) An increase in total protein and globulin concentration and decrease in albumin concentration was recorded in current study (Figure 1) and same pattern was reported previously in horses.(17)

In Figure 2(A&B), the same pattern of antigen-antibody precipitin lines developed in double immune diffusion gel when immunized raw serum of camels and horses allowed to react with selected venoms as reported.(33, 34) Furthermore, faint antigen-antibody precipitating lines were observed in the camel samples against *Daboia russelii* antigens (Figure 2 A), indicating that the camel's antibodies were poorly responsive to *Daboia russelii* venom. Overall, double immune diffusion gel revealed that both horses and camels were responsive to all three viper venoms used in the existing study, even *Daboia russelii* venom, known to contain toxins of high molecular weight and highly immunogenic.(35)

The migration of proteins in horse and camel crude serum was different under non-reducing (Figure 3(A)) and reducing (Figure 3(B)) conditions of SDS-PAGE gel. Camel's serum exhibits two bands of whole IgGs, one around 160 kDa and the other around 100 kDa in non-reducing circumstances, as shown in (Figure 3(A); CCP lane). In contrast, in reducing conditions, the 160 kDa band splits into 50 kDa heavy chains and 30 kDa light chains, while the 100 kDa band splits into 46 kDa and 43 kDa heavy chains as shown in Figure3(B); CCP lane .(36, 37) Our camel's immunized serum also exhibited bands around reported molecular weight proteins as present in Figure 3(B); CCP lane. Under non-reducing circumstances, the immunized crude serum of horses exhibits a single band of the whole IgG at 160 kDa as shown in Figure 3(A); HCP lane. Under reducing conditions, horse IgGs dissociate into a heavy chain (75 kDa) and a light chain (25 kDa) as shown in Figure 3(B);HCP lane.(38) In conclusion, camels and horses positively responded to produce antivenom immunoglobulins against Pakistani viper venoms. Although the physical impact of viper venoms mixed with Freund's complete/incomplete adjuvant was severe in camels compared to horses. Further research is required to clarify this phenomenon.

Limitations

The innovative discoveries were constrained by the lack of modern tools for biochemistry, radiology, and haematology. Furthermore, research on different types of adjuvants is required, which might reduce the lump size at the immunization site, especially in camels.

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Conflicts of interest

The authors declare no financial conflicts of interest or personal ties.

Disclosure

This is an original work carried out at laboratories of Institute of Biochemistry, University of Sindh, Jamshoro and Sindh Anti Snake Venom and Anti Rabies Serology Laboratory, Sakrand.

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