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ASEAN-INDIA COLLABORATIVE RESEARCH PROJECT

"Studies on epidemiology, hospital management of snakebite, and standardization of laboratory tests for assessment of efficacy and quality control of commercial antivenom manufactured in India and in ASEAN countries"

FINAL PROJECT REPORT

(1st February, 2018 to 31st January, 2021)

Submitted By

Prof Ashis K. Mukherjee

Principal Investigator

Department of Molecular Biology and Biotechnology

Tezpur University, Tezpur-784 028, Assam

Section A: Project Details

A1. Project Title	Studies on epidemiology, hospital management of snakebite, and standardization of laboratory tests for assessment of efficacy and quality control of commercial antivenom manufactured in India and in ASEAN countries
A2. DST sanction no. and date	IMRC/AISTDF/R&D/P-3/2017
A3. Name of the PI	Prof. Ashis K. Mukherjee,
A4. Institute	Tezpur University, Tezpur 784028, Assam, India
A5. Address with Contact Nos. (Landline & Mobile) & Email:	Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur 784028, Assam, India Ph: 03712-275405 (O), +917896003886 (M) E-mail: akm@tezu.ernet.in
A6. Total cost	Rupees 48,27,550/-
A7. Duration of report	1st February, 2018 to 31st March, 2021 (3 years)
A8. Approved objectives of the project	**1.** To study the epidemiology of snakebite in some selected districts of Assam, NE India, in Malaysia and in Vietnam. **2.** To investigate the pathophysiology and clinical correlation of snakebite envenomation as well as the effectiveness of snakebite management at district hospitals and tertiary care centers in some selected districts of Assam, NE India, Malaysia, and Vietnam **3**. To develop the standardization of laboratory tests for assessment of efficacy and safety of commercial polyvalent/monovalent antivenom manufactured in India and ASEAN countries.
A9. Specific recommendation by task force, if any	No

Ashis Kr. Mukherjee

Dated: 9 July, 2021 **(A. K. Mukherjee)**

Section-B: Scientific and Technical Progress

B1. Progress made against the Approved Objectives, Targets & Timelines during the Reporting Period (2018-2021)

B1.1. Approved Objective 1: To study the epidemiology of snakebite in some selected districts of Assam, NE India, in Malaysia and in Vietnam.

B1.1.1 Epidemiology of snakebite in some selected districts of Assam, NE India

A hospital based retrospective study of snakebite incidence was conducted from January 2015 to October 2018 at different hospital and health Centre of Sonitpur District, Assam. (Fig.1). A prior consent was obtained from Directorate of Health and family welfare, Govt. of Assam, India for assessing the record room of the hospital. Ethical clearance was obtained from the Institutional Ethical clearance Committee of Tezpur University, Assam.

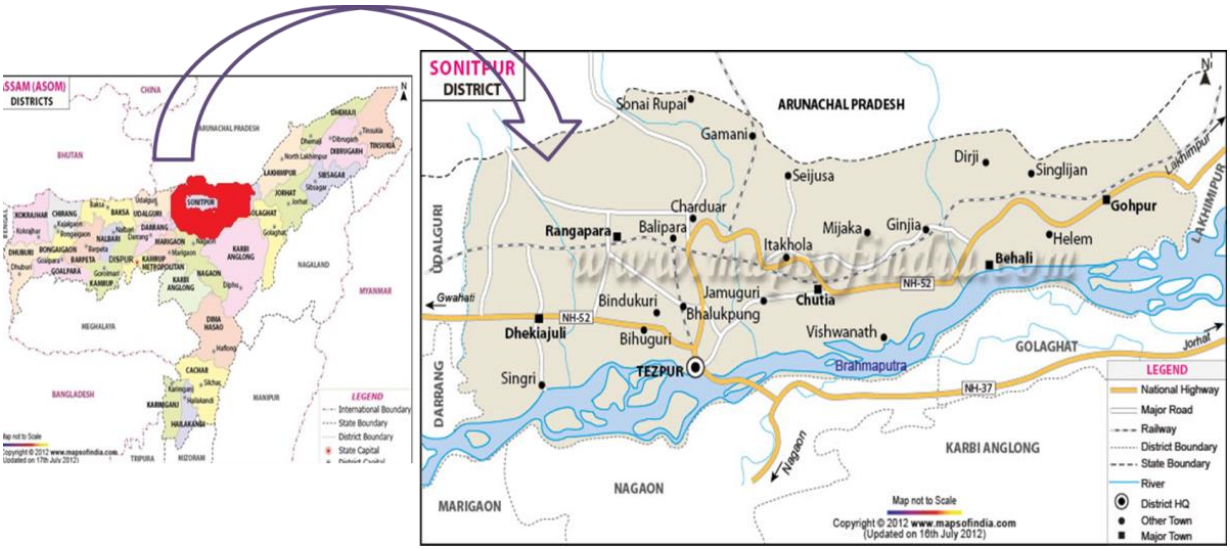


Fig 1: Map of Sonitpur district, Assam (Source- www.mapsofindia.com)

Table1: Year and block wise snake bite incidence in Sonitpur District, Assam.

	No. of snake bite incidents / year			
Block	**2015**	**2016**	**2017**	**2018**
Balipara	27	1	3	0
Dekhiajuli	7	1	0	0
North Jamuguri	7	1	4	1
Bighuguri	18	12	14	0
Behali	20	5	5	1
B.Chariali	3	1	0	0
Gophur	1	1	0	1
Total	83	22	26	3

Total number of snakebite incidence was 134 reported from the Health Centers and hospitals of Sonitpur District, Assam during the period 2015-2018.The year wise incidence of snake bite from different blocks of sonitpur district is mentioned in the Table 1.

The primary limitations of our study were improper maintain of data of snakebite cases in the hospital and in maximum cases the patent was referred to the Guwahati Medical college hospital from block health center for further treatment.

B1.1.2. Epidemiology of snakebite in Vietnam

Vietnam in South-East Asia, with a land cover of 3, 10,000 km2 and a population of 92 million, is a large repository of varied herpetofauna. Of the approximately 140 species of snakes found in Vietnam; 31 species are venomous, of which 18 species are terrestrial and 13 species are marine (Harding and Welch, 1980; Quyen, 2003). The medically important venomous snakes of Vietnam, responsible for fatal bites in this region are shown in Table 2. It is noteworthy that, snakebite burden in other south-east Asian countries like Thailand (Chanhome et al., 1998; Pochanugool et al., 1998; Viravan et al., 1992), Malaysia (Chew et al., 2011; Jamaiah et al., 2006), and Myanmar (Chippaux, 1998; Pe et al., 2006) are well documented. However, snakebite incidences in Vietnam are neither globally or nationally comprehensively surveyed. Only a few hospital-based data on snakebite in Vietnam are readily available. Therefore, we did the analysis of snakebite envenomation in Vietnam.

Vietnam bears witness to approximately 30,000 snakebites annually with a mortality rate of 80 per million per year (Cheng and Winkel, 2001; Warrell, 1999). However, only a few publications in English described the medically important snakes and snakebite burden in Vietnam. The percentage of bites by venomous snakes recorded from different hospital-based surveys is shown in Fig.1. Though the data does not enable a precise projection, but it is evident that the green pit viper is predominantly responsible for most number of bites followed by the cobra and Malay pit viper in some provinces of Vietnam (Fig.2).

The total number of snakebite cases admitted in different hospitals of Vietnam is summarized in Table 3. The data indicates that similar to several other developing countries, poor documentation of snakebite records is a severe problem in Vietnam and it requires immediate medical attention. It may be anticipated the actual snakebite incidence in Vietnam is far more than the published data, since most of the victims depend upon the traditional healers, which are not recorded or tracked in hospital records.

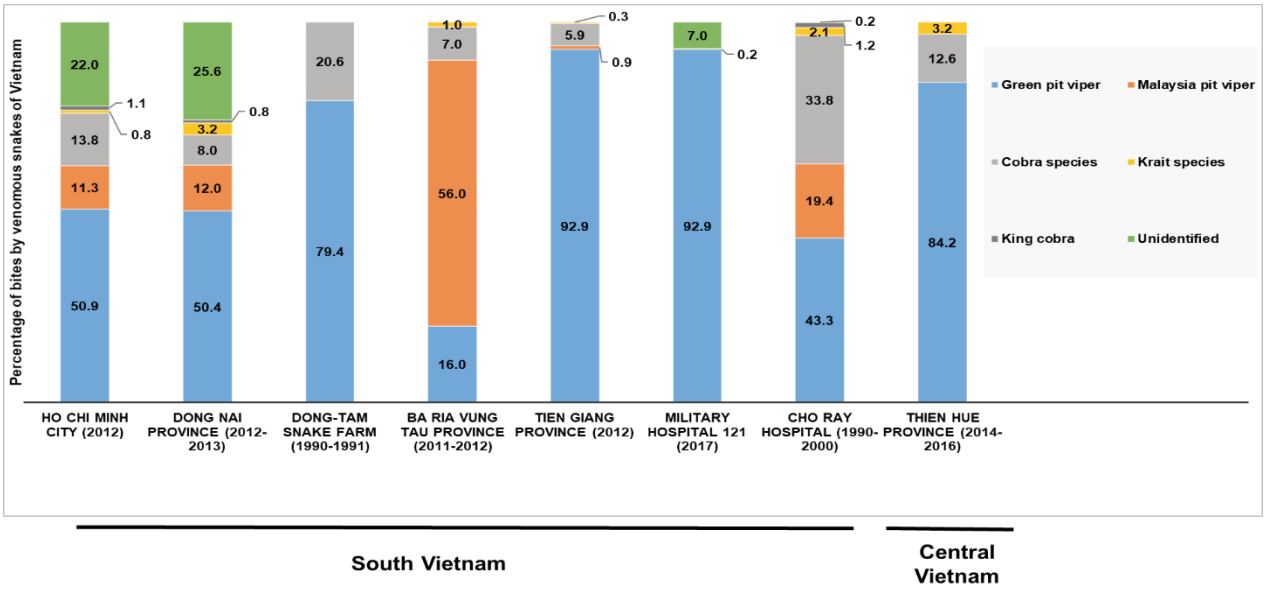


Fig. 2. Analysis of snakebite data in Vietnam covering a period from 1990 to 2020. The percentage of different types of venomous snake bites recorded in different hospitals of Vietnam. (Sources of data: (Berlinger and Flowers, 1973; Blessmann et al., 2018; Dong et al., 2003; Eriksson, 2008, 2011; Eriksson and Nguyen, 2017; Quyen, 2003; Thang et al., 2020)

Table 2: A survey on occurrence and distribution of medically important venomous snakes of Vietnam.

Snake Family	Scientific name	Common name	Distribution	References
Viperidae	*Trimeresurus albolabris*	White-lipped green pit viper	In whole country	(Berlinger and Flowers, 1973; Blessmann et al., 2018; Dong et al., 2003; Eriksson and Nguyen, 2017; Quyen, 2003; Thang et al., 2020)
	Trimeresurus popeorum	Pope's green pit viper	In the Centre and South: Gia Lai, Lam Dong and Ca Mau provinces	
	Calloselasma rhodostoma	Malayan pit viper	In the South: Ninh Thuan, Binh Duong, Binh Phuoc, Tay Ninh, Dong Nai, Ba ria-Vung Tau, Binh Thuan and An Giang provinces	
Elapidae	*Naja kaouthia*	Monocellate cobra	In South Vietnam	
	Bungarus candidus	Malayan krait	In Centre and South Vietnam: Nghe An, Quang Binh, Thua Thien-Hue, Dac Lac, Lam Dong, Nha Trang, Ninh Thuan, Tay Ninh and Dong Nai provinces	
	Bungarus fasciatus	Banded krait	Whole country	
	Ophiophagus hannah	King cobra	Whole country	
	Hydrophis sp.	Sea snakes	Along with sea sides	

Table 3: Summary of hospital based survey of snakebite incidence reported in different hospitals of Vietnam.

Region	Nam of the hospitals	Year/period of the study	No. of cases	No of Death	Snakes responsible for bites	References
North Vietnam	Bach Mai Hospital	2001-2002	284	3 (2 cobra bites, one unknown bites)	Not reported	(Eriksson, 2008, 2011)
Central Vietnam	Central Hospital and 9 district hospital at Thien Hue province	2014-2016	221	No deaths was reported	Snakes were identified for 95 cases. Green pit vipers- 80; Cobra bites- 12; krait bites-3	(Blessmann et al., 2018)
	Da-Nang No17 Hospital	1991	84	0	Cobra- bites-64; rests by non-venomous snakes	(Quyen, 2003)
South Vietnam	Institut Pasteur, SV (SV)	1948-1954	124	2	All were reported as cobra bites	(Swaroop and Grab, 1954)
	Dong-Tam Snake Farm (SV)	1990-1991	391	12 (all were from cobra bites)	Green pit viper- 250 Cobra bites- 65 Non-venomous snakes- 76	(Quyen, 2003)
		1995-1996	64	15	All were reported as cobra bites	
	Cho ray Hospital (SV)	1990-2000	3867	59	Deaths were caused by *B. candidus, N. siamensis, N.*	(Quyen, 2003)

					kaouthia and *C. rhodostoma* bites	
	Paediatric Hospital from the Military Hospital 121	2017	450 (435 from Military Hospital 121; 15 from Paediatric hospital)	0	Green pit viper bites- 414 Cobra bites -1 Not identified- 31	(Thang et al., 2020)
	Dong Tam Clinic, Dong Tam Snake Farm	2012	4690	No deaths reported	Only data of 2012 was available Green pit viper bites- 658 Malaysia pit viper- 6 Cobra bites -42 Bungarus fasciatus-2	(Eriksson and Nguyen, 2017)
	Ba Ria Provincial Hospital	2011-2013	154	1	Green pit viper bites- 28 Malaysia pit viper-75 Cobra bites -9 Bungarus sp.-1 Unidentified-41	(Eriksson and Nguyen, 2017)
	Binh Phuoc Provincial Hospital	2011	39	No deaths recorded	Data was not available	(Eriksson and Nguyen, 2017)
	Dong Nai Province Hospital	2012-13	125	Data was not available	Green pit viper - 63 Malaysia pit viper -15	(Eriksson and Nguyen, 2017)

					Cobra -10 King cobra- 1 Banded krait- 4 Unidentified-32	
	Cho Ray Hospital, Ho Chi Minh City	2011-12	1808	Data was not available	Green pit viper - 920 Malaysia pit viper -205 Cobra -250 King cobra- 20 krait- 15 Other snakes 398	(Eriksson and Nguyen, 2017)

Table 4: Venomous snakes of Malaysia: their distribution and clinical manifestation upon envenomation

No.	Species	Common name	Locality	Clinical symptoms		References
				Systemic symptoms	**Local symptoms**	
	Elapidae			Krait envenoming may cause a sudden or delayed onset of rapidly progressive respiratory paralysis with minimal local manifestation	No pain at bite site and minimal local tissue damage.	(Das et al., 2015; Ismail, 2015)
1.	*Bungarus candidus*	Malayan krait	Peninsular Malaysia (Common)			
2.	*Bungarus fasciatus*	Banded krait	Peninsular Malaysia (Common), Sarawak (Common)			
3.	*Bungarus flaviceps*	Red-headed krait	Peninsular Malaysia (Not common), Sarawak (Not common)			
4.	*Calliophis bivirgatus*	Blue coral snake	Peninsular Malaysia (Common), Sarawak (Common)	The destruction of striated muscles frequently manifests as myalgia, muscle stiffness,dark-colored urine, or myoglobinuria. Acute kidney injury and severe hyperkalemia may occur secondary to major myolysis	edema may occur sometimes but otherwise not very obvious	
5.	*Calliophis gracilis*	Spotted coral snake	Peninsular Malaysia (Not common)			
6.	*Calliophis intestinalis*)	Striped coral snake	Peninsular Malaysia (Common), Sarawak (Common)			
7.	*Calliophis maculiceps*	Speckled coral snake	Peninsular Malaysia (Not common)			

8.	*Naja kaouthia*	Monocled cobra	Peninsular Malaysia (Common)	Acute neurological dysfunction with ptosis, phthalmoplegia, dysphagia, aphasia, hypersalivation, and respiratory paralysis	Local tissue necrosis with severe tissue damage and edema	
9.	*Naja sumatrana*	Sumatran spitting cobra	Peninsular Malaysia (Common), Sarawak (Common)			
10.	*Ophiophagus hannah*	King cobra	Peninsular Malaysia (Common), Sarawak (Common)			
	Viperidae					
11.	*Calloselasma rhodostoma*	Malayan pit viper	Peninsular Malaysia, northern states Common)	Vascular effects such as precipitous hypotension, bleeding, and indirect hemolysis. Life-threatening coagulopathy can cause bleeding from the bite site, gingival sulci, and venepuncture sites as well as from visceral organs	Progressively worsening pain, swelling, blisters, and subsequent necrosis are common.	
12.	*Cryptelytrops venustus*	Beautiful pit viper	Peninsular Malaysia, northern states (Not common)			
13.	*Cryptelytrops purpureomaculatus*	Mangrove pit viper	Peninsular Malaysia (Common)			
14.	*Garthius chaseni*	Kinabalu brown pitviper	Sabah -HA, endemic to Borneo			
15.	*Ovophis convictus*	Malayan brown pitviper	Peninsular Malaysia (Common)			
16.	*Parias hageni*	Hagen's green pitviper	Peninsular Malaysia (Common)			
17.	*Parias malcolmi*	Kinabalu green	Sabah -HA,			

		pitviper	endemic to Sabah			
18.	*Parias sumatranus*	Sumatran pit viper	Peninsular Malaysia -(Common), Sarawak -(Common)			
19.	*Popeia buniana*	Pulau Tioman pitviper	Peninsular Malaysia, endemic to PulauTioman			
20.	*Popeia fucata*	Thai Peninsular pitviper	Peninsular Malaysia (Common)			
21.	*Popeia nebularis*	Cameron Highlandspit viper	Peninsular Malaysia (Common), endemic			
22.	*Popeia sabahi*	Sabah green pit viper	Sarawak -HA, endemic toBorneo			
23.	*Trimeresurus borneensis*	Bornean palm pitviper	Sarawak C, endemic to Borneo			
24.	*Trimeresurus wiroti*	Wirot's palm pit viper	Peninsular Malaysia (Common)			
25.	*Tropidolaemus wagleri*	Wagler's pit viper	Peninsular Malaysia (Common)			
26.	*Tropidolaemus subannulatus*	Bornean pit viper	Sabah (Common)			

B1.1.3. Epidemiology of snakebite in Malaysia:

Snakebite is a severe problem in Malaysia like other tropical countries. A variety of medically important venomous snakes are recorded from Malaysia. The medically important venomous snakes and their distribution are shown in Table 4. Studies have shown that Malaysia witnessed 400–650 snakebites per 100,000 populations per year and with a mortality rate of 0.2 per 100,000 populations per year (Chippaux, 1998; Kasturiratne et al., 2008). Ministry of Health (MOH), in Malaysia reported the 4,024 cases and 3,658 cases in 2009 and 2011 respectively (Das et al., 2015).

B1.2. Approved Objective 2: To investigate the pathophysiology and clinical correlation of snakebite envenomation as well as the effectiveness of snakebite management at district hospitals and tertiary care centers in some selected districts of Assam, NE India, Malaysia, and Vietnam

B1.2.1. Clinical feature of snakebite in NE-India

Out of 102 species found in North-East India, approximately 18 species belonging to the families of Viperidae and Elapidae. Most of the envenomation are attributed to monocled cobra (*Naja kaouthia*), black krait (*Bungarus niger*) and green pit vipers (*Trimeresurus albolabris*) and mountain pit vipers (*Ovophis monticola*). Clinical manifestations of the medically important venomous snakes found in North-Eastern India including Assam are shown in Table 5.

B1.2.2 Snakebite management in North-Eastern India

Although antivenom is the only available choice of treatment for snakebite envenomation; however, many people still now rely on the traditional healer (Personal communication with healthcare staff at primary health centre and local people). The standard protocol for snakebite treatment begins with identification of snake species. Due to lack of commercial diagnostic kits (Puzari and Mukherjee, 2020), the identification of snake species depends upon experience of the clinicians and symptoms of envenomation such as fang marks, local swelling, spontaneous systematic bleeding from gums, respiratory failure, cardiac arrest and/or neuromuscular paralysis, which guide the physician to predict the species of snake involved. Sometimes, common laboratory investigations, such as complete blood count (CBC), whole blood clotting test (WBCT), prothrombin time (PT), international normalized ratio (INR), renal function test (RFT) and liver function test (LFT) are also used to confirm if viper envenomation is involved; and neurotoxic bites are confirmed by clinical judgement for elapid bites (Mukherjee et al., 2020).

Most of the primary health centre do not have antivenom in stock and facilities to treat snake envenomed patients (Personal communication with doctors at primary health care centre). The primary healthcare centre usually refers the patient to the district civil hospital or medical college hospital. We visited Tezpur Medical College Hospital and Kanaklata Civil Hospital and the doctors at the hospitals reported the antivenom is not always available in the hospital and they refer the snake envenomation cases to Guwahati Medical College and Hospital. The antivenom available in the Tezpur Medical College Hospital and Kanaklata Civil Hospital manufactured by Bharat Serum and Vaccines Pvt. Ltd., Hyderabad, India. The other antivenoms available in the market of Sonitpur district and Kamrup district of Assam was manufactured by VINS Bioproducts Ltd, Mumbai, India and Virchow Biotech Ltd, Hyderabad India.

Table 5: Medically important snakes in Assam, North-East India and their pathophysiology upon envenomation

Snake species	**Geographical Distribution**	**Clinical symptoms**		**References**
		Systemic symptoms	**Local symptoms**	
Naja kaouthia	Throughout North-Eastern India, Eastern India and Northern India	Blurring of vision, loss of consciousness, early neuroparalysis, and rapid onset of respiratory failure	severe pain at the bite site, rapid progression of swelling, local tissue damage due to the large, non-healing ulcer formation	(Chanda et al., 2018) (Mukherjee et al., 2020)
Bungarus niger	Assam, Arunchal Pradesh, Nagaland, Meghalaya, Mizoram, North Bengal and Sikkim	Neuromuscular paralysis, blurring of vision, loss of consciousness, myalgia, myotoxicity	No pain at bite site, no local tissue damage occur	(Faiz et al., 2010)
Bungarus fasciatus	Assam, Arunchal Pradesh, Nagaland, Meghalaya,	Neuromuscular paralysis, blurring of vision, loss of consciousness, myalgia, myotoxicity	No pain at bite site, no local tissue damage occur	(Pe et al., 1997)

	North Bengal and Sikkim			
Ovophis monticola	Assam, Arunchal Pradesh, Nagaland, Meghalaya, North Bengal and Sikkim	Consumption coagulopathy and chronic pain, thrombocytopenia	throbbing pain, local swelling, blister formation, necrosis,	(Pandey et al., 2021)
Trimeresurus albolabris	Assam, Arunchal Pradesh, Nagaland, Meghalaya, North Bengal and Sikkim	Consumption coagulopathy , thrombocytopenia, chronic pain	Severe pain at bite site, swelling, tissue necrosis, blister	(Pandey et al., 2019)

The antivenom available in India as well as in Assam is raised against the 'Big four' venomous snakes of India; however, the medically important snakes responsible for envenomings in Asssam are beyond the 'Big four' snakes of India. Therefore, urgent need for development of region-specific antivenom for Assam as well as other North-Eastern states for the improvement of snakebite treatment in this region.

B1.2.3. Clinical features of snakebites in Vietnam

Detailed studies on clinical features of snakebite in Vietnam are lacking and/or not well documented. Only few discrete studies have demonstrated the clinical symptoms following venomous snake bites in Vietnam, summarized in Table 6.

B1.2.4. Snakebite management in Vietnam

Although antivenom is the only approved treatment by WHO against snake envenomation; however, people of Vietnam mostly rely on the traditional healers, due to unavailability of antivenom in hospitals, high cost of antivenom treatment, and/or lack of social awareness (Eriksson, 2011; Eriksson and Nguyen, 2017).

In Vietnam, no nation specific standardized protocol is available or developed for the clinical management of snakebite. The treatment procedure of snakebite in Cho Ray Hospital, Ho Chi Minh

City has been described in detail (Quyen, 2003). Antibiotic (amoxicillin, 1.5g per day and/or ciprofloxacin, 1.5 g per day) was administered to patient to reduce the severity of wound infection caused by snakebite. Benzyl penicillin with one mega-unit and aminoglycoside such as gentamicin were given by intravenous injection for severe cases. The third generation cephalosporin family was administered when the patients were supported by artificial ventilation. To balance the level of water and electrolytes, fluid transfusion was also used and it was thought to be helpful for elimination of venom from the circulatory system, although the quantum of venom in the blood could not be determined. The patients showing coagulation disturbance were transfused with fresh plasma and platelets. The patients who developed severe hemorrhage were treated with transfusion of fresh whole blood or red blood cells. Due to the limited availability of antivenom in the hospital, it was administered only to severe patients. However, there is no mention of the number of vials of antivenom administered to patients, adverse reactions of antivenom therapy, if any, and the treatment given to patient to reduce the serum reactions. The non-antivenom treatment cases were treated with supportive and symptomatic therapy (Quyen, 2003).

The antivenom manufactured and available in different hospitals of Vietnam is shown in Table 7. The monovalent antivenom (MAV) is prevalent in Vietnam, though there are several major issues related to antivenom treatment in this country. First, MAV is superior to polyvalent antivenom (PAV) in treating snakebite patient, albeit administration of MAV requires proper diagnosis and correct identification of the species that caused the envenomation by the clinician. The second issue is that the production rate of antivenom in Vietnam is far below the national requirement, resulting in most hospitals being devoid of antivenom, thus obstructing effective management of snakebite (Eriksson and Nguyen, 2017). Moreover, the cost of antivenom, if available, is also beyond the economic capacity of the common people. Therefore, rural poor people in Vietnam, who are predominantly affected by snake bite, are deprived of costly treatment, leading to high snakebite mortality and morbidity in Vietnam (Eriksson and Nguyen, 2017). Thus, large rural populations far away from hospitals, lack of trained clinician and/or medical staff to treat snakebite, limited medical resources at district and rural hospitals, unavailability and/or high cost of antivenom, lack of snakebite education, and social awareness are the foremost obstacles for a proper clinical management of snakebite in Vietnam.

Table 6: Clinical symptoms of venomous snakebites in Vietnam.

Name of the snakes	**Clinical symptoms**		**References**
	Local symptoms (Percentage of patients showing the symptoms)	**Systemic envenomation (Percentage of patients showing the symptoms)**	
Trimeresurus albolabris (white lipped green pit viper)	▪ Oozing of blood from the bite site (15.9%), ▪ Blister (18.9%), and ▪ Necrosis (8.1%)	▪ The spontaneous systemic symptoms were as follows haemorrhage (48.6%) petechiae (35.1%),gingival sulci (8.1%), haematuria (2.7%); and abnormal menstruation (2.7%). ▪ In addition, gastrointestinal symptoms are shown as vomiting (21.6%), nausea (21.6%), diarrhoea (13.5%) and colic pain (13.5%) ▪ Thrombocytopenia was seen in 16.2% patients	(Quyen, 2003)
Calloselasma Rhodostoma (Malayan pit viper)	▪ Severe pain, swelling, bleeding from the site of the bite and many blisters were common local symptoms. Bleeding from the bite site (77.5%), blistering in the bitten limbs (62.5%). The bleeding from the broken blisters was life threatening and sometimes led to the death .Immediately severe pain was observed in 70% of the cases.	▪ The predominant signs of coagulation disorder caused by *C. rhodostoma* bite were spontaneous systemic haemorrhages. In skin, multiform haemorrhages viz. petechiae and ecchymoses were seen in (45%), and purpura in (62.5%). ▪ Bleeding was also seen from the sites of venepunctures and intramuscular injection (65%); from gingival sulci (35%); gastrointestinal tract (15%), kidneys (haematuria) (17.5%) and female genital organ (abnormal menstruation) (22.2%)	(Quyen, 2003)

	▪ Necrosis at the site of bite was 17.5%.	▪ Haemorrhage of brain was observed 10%. In these cases, patients developed symptoms of neurological disorder such as coma, dilated pupil and neck stiffness as well as non-specific systemic symptoms such as syncope (12.5%), vertigo (32.5%), dyspnoea in (42.5%), weakness (75%) and acute anaemia in (40%). ▪ Thrombocytopenia was observed in 83% cases.	
Naja kaouthia (Monocled cobra)	▪ Severe Pain was observed in at the bite site in 88.2% cases ▪ Necrosis at the site of bites was seen in 76.5% patients. ▪ No patients had blisters at the bitten limbs ▪ Regional lymph node was impalpable. The swelling was seen in most of the patients	▪ Neurotoxic envenomation syndrome was predominantly seen in N. kaouthia envenomation ▪ Blurred vision 82.4%; “heavy” of eyelids 70.6% ; numbness of lips 64.7%; ptosis (70.6%) patients (around 2 hours after the bites) were the primary symptoms. ▪ 52.9% patients could not open their mouth and 76.5% of them had dysphonia. ▪ Hypersalivation and dysphasia was observed in 52.9% 58.8% cases respectively. ▪ The primary symptom of neurotoxic envenomation was respiratory paralysis observed in 58.8% patients within four hrs after the bites and artificial ventilation was used to support the respiratory failure. ▪ No haemorrhagic signs were observed however, vomiting (29.4%); abdominal pain (11.8%); headache (11.8 %) dizziness (47.1%) and muscle pain (41.2%) were seen.	(Quyen, 2003)

Naja bites (Naja atra or *Naja Kaouthia)*	▪ All patients had developed swelling, necrosis, and local pain typical of *Naja* spp bites	▪ Mild to severe neurotoxic symptoms were observed	(Ngo et al., 2020)
Naja siamensis (Indo-Chinese slitting cobra)	▪ Fang marks were frequently observed in all patients. ▪ Severe pain was observed in 64.7% patients ▪ Swelling occurred quickly in all of the patients. ▪ Blisters and necrosis at bite site and was seen 11.8% 88.2% cases respectively. ▪ Some patients (52.9%) had lymph node enlargement. ▪ Two patients (out of 17) had venom spat in their eyes. Conjunctivitis, pain and swelling of eyes were observed but no corneal ulcers was seen.	▪ Myalgia and gastrointestinal symptoms were the predominant symptoms. ▪ The patients were suffering from generalized pain, especially in the muscles (82.4%). ▪ Weakness was also seen in 70.6%. ▪ Vomiting (52.9%), diarrhoea (47.1%), colic pain (52.9%), headache (23.5%) and vertigo (17.6%) was observed. Tachycardia was seen in 17.6% but none of them had arrhythmia. ▪ Acute renal failure with oliguria on admission was developed in 11.8% ▪ One of these patients (out of 17) also developed coagulation disorder but no bleeding at venepuncture sites. ▪ 23.6% bite patients have fever. Unconsciousness was observed in three patients due to fever and shock; however, after treatment, the consciousness returned. ▪ Unlike *N. kaouthia*, the neurotoxic symptoms were not seen in patients, bitten by *N. siamensis*. None of	

		patients had ptosis but two patients informed about blurred vision.	
Bungarus candidus (Malayan krait)	▪ Minimal local reaction was observed. None of them was suffering from pain; however, two patients had numbness. ▪ No swelling nor necrosis was observed. ▪ Bleeding from bites was not seen.	▪ Neurotoxic signs and symptoms were predominant in most patients. ▪ After an hour of bites, numbness on lips and tongue, and dysphasia was seen in three patients (out of 7). ▪ Around 2-4 hours, bilateral ptosis was observed in all cases. Difficulties was observed in opening mouth, to cough or to expectoration. Further, hypersalivation, dysphonia, breathlessness and dilated pupils were observed in all patients. ▪ Respiratory paralysis was commonly seen within 4 hours after the bites. Most of the patients required tracheal intubation. ▪ Patients were conscious but could not perform doctor's commands because of muscle paralysis. ▪ Hypertension was observed in two cases (out of 7) after 24 hours using artificial ventilation. The other one was recorded shock and required to use vasoconstrictors. ▪ Secondary pneumonia was developed due to prolonged respiratory failure.	(Quyen, 2003)
Bungarus candidus (Malayan krait)	▪ Fang marks were distinguishable at the bite site	▪ Signs of systemic neurotoxicity was observed in all patients in which 74.1% was life-threatening.	(Trinh et al., 2010)

	▪ Local numbness was observed in 35.7% patients. ▪ Swelling was not observed in any cases.	▪ Thirty patients (71.4%) required endotracheal intubation and rests were mechanically ventilated. ▪ Myalgia was not observed in this case series. ▪ Pupillary dilatation was seen in all patients. Further, Pupillary dilatation was remained even after discharge of patients, when they were followed up.	
Bungarus Multicinctus (Many banded krait)	▪ Pain, swelling, necrosis was not observed in any patients	▪ The neuromuscular symptoms and signs most commonly observed which are ptosis (12%), mydriasis, ophthalmoplegia, jaw weakness, pharyngeal pain, palatal palsy, general myalgia (9%), neck muscle paralysis, paralysis of the extremities (most pronounced proximally), absent or diminished tendon reflexes, paralysis of the respiratory muscles, urinary retention, and decreased bowel movements	(Hung et al., 2010)
Ophiophagus hannah	▪ Bite site was punctured by single marks or double marks and sometime scratches. ▪ Patients were suffered from severe pain ▪ Immediate swelling was seen. ▪ Necrosis was not observed in this series of cases.	▪ Signs and symptoms of neurotoxic envenoming was not seen in any cases. ▪ Coagulation disorder was also not observed.	(Quyen, 2003)

Table 7: Commercial antivenom producers in Vietnam.

Name of the antivenom product	**Raised against**	**Production Unit and address**	**Source of the Information**
SAV-*Naja*	*Naja spp.*	Institute of Vaccines and Biological Substances, Nha Trang, Vietnam	WHO, WCH-Toxinology
SAV-*Trimeresurus*	*Trimeresurus spp.*	Institute of Vaccines and Biological Substances, Nha Trang, Vietnam	WHO, WCH-Toxinology
Bungarus candidus Antivenom	*Bungarus candidus*	Venom Research Unit, University of Medicine and Pharmacy Ho Chi Minh City, Vietnam	WCH-Toxinology
Calloselasma rhodostoma - Malayan Pit Viper Antivenom	*Calloselasma rhodostoma*	Venom Research Unit, University of Medicine and Pharmacy Ho Chi Minh City, Vietnam	WCH-Toxinology
Naja kaouthia Antivenom	*Naja kaouthia*	Venom Research Unit, University of Medicine and Pharmacy Ho Chi Minh City, Vietnam	WCH-Toxinology
Naja siamensis Antivenom	*Naja siamensis*	Venom Research Unit, University of Medicine and Pharmacy Ho Chi Minh City, Vietnam	WCH-Toxinology
Ophiophagus hannah Antivenom	Ophiophagus hannah	Venom Research Unit, University of Medicine and Pharmacy Ho Chi Minh City, Vietnam	WCH-Toxinology
B. candidus monospecific antivenom	*Bungarus candidus*	Venom research & Antivenom production Unit, The National Poison Control Center, Bach Mai Hospital, Hanoi, Viet Nam	(Trinh et al., 2010)

B1.2.5. Clinical features of snakebites in Malaysia

The clinical manifestation of medically important snakes of Malaysia is shown in Table 4. Further, detailed region specific clinical symptoms of a particular snake species should be investigated.

B1.2.6. Snakebite management in Malaysia

Like other countries antivenom is the only choice of treatment for snakebite treatment in Malaysia. Monovalent antivenoms are mostly used in Malaysia; therefore, use of specific antivenom should be decided by a physician or clinical toxinologist who are familiar and experienced with snakebite and envenomation management. The antivenoms available in Malysia is shown in Table 8.

Table 8 Recommended selection of antivenom appropriate for use in Malaysia (Ismail, 2015)

	Species raised from and manufacturer	**Coverage area**	**First dose/vial**
1.	Monocle cobra, *Naja kaouthia*	Peninsular Malaysia, Sabah, & Sarawak	100 ml/10 vials
	QSMI Thai Red Cross: cobra antivenin to neutralize 0.6 mg/ml of venom		Subsequent dose 1–2 h
2.	King Cobra, *Ophiophagus hannah*	Peninsular Malaysia, Sabah, & Sarawak	100 ml/10 vials
	QSMI Thai Red Cross: king cobra antivenin to neutralize 0.8 mg/ml of venom		Subsequent dose 1–2 h
3.	Malayan krait, *Bungarus candidus*	Peninsular Malaysia	50 ml/5 vials
	QSMI Thai Red Cross: Malayan krait antivenin to neutralize 0.4 mg/ml of venom		Subsequent dose 1–2 h
4.	Banded krait, *Bungarus fasciatus*	Peninsular Malaysia, Sabah, & Sarawak	50 ml/5 vials
	QSMI Thai Red Cross: banded krait antivenin to neutralize 0.6 mg/ml of venom		Subsequent dose 1–2 h
5.	Malayan pit viper, *Calloselasma rhodostoma*	Peninsular Malaysia	40 ml/4 vials
	QSMI Thai Red Cross: Malayan pit viper antivenin to neutralize 1.6 mg/ml of venom		Subsequent dose 6 h
6.	Green pit viper, *Cryptelytrops albolabris*	Peninsular Malaysia, Sabah, & Sarawak	30 ml/3 vials
	QSMI Thai Red Cross: green pit viper antivenin to neutralize 0.7 mg/ml of venom		Subsequent dose 6 h

7.	Malayan pit viper, *Calloselasma rhodostoma*; green pit viper, *Cryptelytrops albolabris*; SEA Russell's viper, *Daboia siamensis*	Peninsular Malaysia	30 ml/3 vials
	QSMI Thai Red Cross: hemato-polyvalent snake antivenom		Subsequent dose 6 h
8.	Monocled cobra, *Naja kaouthia*; king cobra, *Ophiophagus hannah*; banded krait, *Bungarus fasciatus*; Malayan krait, *Bungarus candidus*	Peninsular Malaysia, Sabah, & Sarawak	50–100 ml/5–10 vials
	QSMI Thai Red Cross: neuro-polyvalent snake antivenom		Subsequent dose 1–2 h
9.	Sea snakes, *Hydrophiinae*	Peninsular Malaysia, Sabah, & Sarawak	10–30 ml/1–3 vials
	CSL, Australia: polyvalent sea snake antivenom		Subsequent dose 1–2 h

Note: Subsequent repeat doses are according to the clinical signs and symptoms. Antivenom for monocled cobra, *Naja kaouthia*, has paraspecific properties and provides good cross neutralization to the venom of equatorial spitting cobra, *Naja sumatrana*. Malayan pit viper, *Calloselasma rhodostoma*; Malayan krait, *Bungarus candidus*; and monocled cobra, *Naja kaouthia* are not indigenous to Borneo. Southeast Asian Russell's viper, *Daboia siamensis*, is not indigenous to Malaysia

Approved Objective 3: To develop the standardization of laboratory tests for assessment of efficacy and safety of commercial polyvalent/monovalent antivenom manufactured in India and ASEAN countries.

B1.3.1. Materials:

Antivenom: Lyophilized equine polyvalent antivenoms (PAVs) raised against *Naja naja*, *Daboia russelii*, *Bungarus caeruleus*, and *Echis carinatus* were obtained from Bharat serum and Vaccines Ltd. (BSVL), India (Batch 1 (B1) - A05315029, expiry date: January 2019; Batch 2 (B2) – A05319007, expiry date: October 2022), Premium Serum and Vaccines Pvt. Ltd. (PSVPL), India (Batch 1- 012015, expiry date: December 2018; Batch 2- ASVS (I) ly- 014, expiry date: December 2022), Virchow Biotech Pvt. Ltd. (VBPL), India (Batch 1- 012005, expiry date: May 2018; Batch 2- PAS00316, expiry date: January 2020), VINS Bioproducts Ltd. (VINS), India (Batch 1- 01AS18026, expiry date: April, 2022; Batch 2- 01AS18020, expiry date: March 2022), and Biological E Limited (Bio-E), India (Batch no. A1604216, expiry date: March 2018).

Venom samples: Samples of *Daboia russelli* venom (DRV) and *Naja naja* venom (NNV) of western India (WI) and eastern India (EI) origin were procured from Haffkine Institute, Mumbai and Calcutta Snake Park, Kolkata, respectively. *N. kaouthia* venom (NKV) from EI origin was also obtained from Calcutta Snake Park, Kolkata. Venoms of *Echis carinatus* (ECV) and *Bungarus caeruleus* (BCV), DRV and NNV from Irula Snake Catchers Association, Chennai (southern India, SI) were kind gifts from Premium Serum and Vaccines Pvt. Ltd., Pune.

All the chemicals and reagents used are of analytical grade and from Sigma-Aldrich, USA. Purified Horse IgG was obtained from Biorad (USA) and purified Horse $F(ab')_2$ were obtained from Jackson ImmunoResearch Inc. (USA).

B1.3.2. Standardization of laboratory tests for assessment of safety of commercial polyvalent antivenom manufactured in India

B1.3.2.1. Physiochemical characterization of Indian PAVs

The appearance of the PAVs (i.e., colour, texture, and uniformity of the product) were tested by visual observation. Before the assay, the dry weight of each vial of PAV was measured. The solubility of the PAVs was tested by examining the cloudiness of the solution after dissolving the whole content in 10 ml of sterilized de-ionized water (as per the recommendation of the manufacturer). The solubility of the PAVs was also tested by determining the presence of any insoluble component(s) after centrifugation at 10,000 rpm for 10 minutes, carefully decanting the solution, drying, and then weighing the amount of insoluble material, if any. The pH of each PAV solution was measured with a digital pH meter (Eutech Instruments, pH 510, USA). Residual moisture content of the PAVs was determined by the loss on drying method by heating the PAV at 105 ºC for 3 h in an oven (Ahn et al., 2014).

The Indian PAVs containing $F(ab')_2$ molecules are marketed as lyophilized (BSVL, PSVPL, VBPL, and VINS) or in liquid form (Bio-E). The lyophilized PAVs had a white, homogenous appearance, though some evidence of macroscopic collapse could be seen with shrinkage and adhesion to the vial (Fig. 3). PSVPL batch 1, VBPL batches 1 and 2, and VINS batch 1 appeared as a fragmented cake, possibly because of some mechanical damage that occurred during the transportation of the product (Herrera et al., 2017). These structures may have some impact on the products since low porosity and poor rehydration properties could affect the reconstitution time, turbidity, and particle formation (Adams et al., 1993). At the same time, however, these structures have no effect on the biological activity of lyophilized products (Wang et al., 2004).

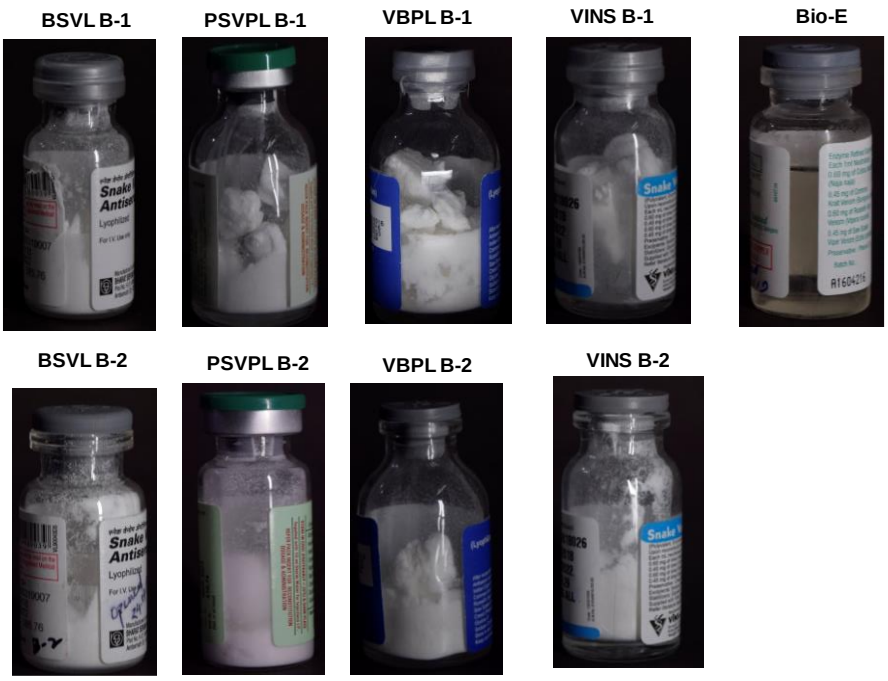


Fig. 3. Physical appearance of PAVs. Images are taken by using digital camera (Nikon D5300). B-1 and B-2 represent the batch 1 and batch 2 of the PAVs, respectively manufactured by the same company (BSVL- Bharat Serum and Vaccines Pvt. Ltd.; PSVPL- Premium Serum And Vaccines PVT. Ltd, VBPL- Virchow Biotech Pvt. Ltd.; .VINS- VINS Bio products Ltd.)

Based on the manufacturers' recommendations, freeze-dried PAV should be reconstituted into 10 mL of sterile deionized water (provided with the PAV). The freeze-dried PAVs should be dissolved within 10 minutes after mixing with sterile water (WHO, 2018). The tested PAVs were found to be solubilized within 10 minutes and they did not contain insoluble material. The PAVs, which are prepared by digesting the horse plasma with pepsin at a lower pH (3.0-3.5), should be adjusted to neutral pH in the final product (WHO, 2018). The pH of the PAV solutions we used were measured to be in the range of 5.9-7.1 (Table 9). According to WHO guidelines, the aqueous solution of antivenom should be at neutral pH (7.0 ± 0.5); however, some manufacturers prefer to formulate the product at a slightly acidic pH to increase its stability as well as to prevent it from aggregation (WHO, 2018). For caprylic acid precipitation, pH of the plasma solution was lowered to 5.5 followed by which the pH of the final product should be adjusted near to a neutral value (WHO, 2018). However, the pH of the final formulation should not exceed 7.5, as higher pH favoured the aggregation of $F(ab')_2$ molecules (WHO, 2018). Therefore, utmost care should be taken to adjust the pH of the final product to neutral.

Table 9: pH of the aqueous solution of PAVs. The values are mean ± S.D. of triplicate determinations.NA: Not available

PAVs	pH of PAV solutions	
	batch 1	batch 2
BSVL	6.88 ± 0.25	7.09 ± 0.15
PSVPL	6.95 ± 0.32	7.02 ± 0.21
VBPL	6.30 ± 0.32	6.05 ± 0.30
VINS	6.05 ± 0.24	5.92 ± 0.32
Bio-E	6.91 ± 0.29	NA

B1.3.2. Calculation of percent protein per gram dry weight of PAVs

The protein content of each batch of PAV was determined by the method of Lowry et al. (1951) and the results were expressed as quantity of protein per gm dry weight of antivenom. The dry weight of the lyophilized powder and percent protein content in each vial is shown in Table 10. The estimation of total protein content offers an idea about the quantity of total antibody or percent of protein content in per gram dry weight of PAVs. The antivenoms contain more than 85% protein and rest content may be the preservative (cresol) and/or excipient (for example, NaCl).

Table 10: Dry weight (g) of the lyophilized powder and percent protein content in each batch of PAVs. The PAV manufactured by Bio-E is a liquid. The weights are mean ± S.D. of contents in three vials.

PAVs	dry weight (g)		Protein content (%)	
	batch 1	batch 2	batch 1	batch 2
BSVL	0.985 ± 0.2	0.992 ± 0.15	92.7 ± 3.8	90.6 ± 4.4
PSVPL	1.036 ± 0.18	1.124 ± 0.21	90.9 ± 2.5	91.8 ± 2.3
VBPL	0.995 ± 0.14	0.882 ± 0.17	88.0 ± 3.2	89.6 ± 3.2
VINS	0.902 ± 0.21	0.965 ± 0.24	86.8 ± 3.6	93.6 ± 2.4
Bio-E	NA	NA	89.6 ± 4.2	-

B1.3.3. Mass spectrometry (LC-MS/MS) analysis to determine the PAV composition and presence of contaminating protein, if any:

The two different batches of PAVs were mixed at 1:1 ratio (protein content) and 100 µg of each mixture of PAVs (protein content) were reduced (10 mM dithiothreitol), alkylated (55 mM iodoacetamide), and then subjected to trypsin digestion for overnight (~16 h) at 37˚C with an enzyme: substrate ratio of 1:30 (Patra et al., 2018). The tryptic peptides were desalted and concentrated followed by subjected to LC-MS/MS analysis, as described previously (Patra et al., 2018).

The homogeneity of five Indian PAVs determined by LC/MS-MS analysis revealed a wide variation in IgG content in PAVs, ranging from 78-95%, with the remaining ~5-20% protein representing degraded products of horse plasma fibrinogen, haptoglobin, alpha-2-macroglobulin, fibronectin, seprin, nexin, and albumin (Figs. 4a-e). This suggests that the PAVs under study are slightly to moderately contaminated with non-IgG proteins (Figs. 4a-e). Nevertheless, by SDS-PAGE analysis and gel-filtration chromatography, these contaminating proteins were not detected (see below) since they were digested by pepsin during the $F(ab')_2$ preparation and the degraded peptide fragments would travel along with the dye font in SDS-PAGE. The peptide fragments were also not detected at 280 nm in the gel filtration chromatography fractions, because this wavelength is not suitable for measuring the elution of peptides devoid of aromatic amino acid(s). Our study suggests that utmost care should be taken in the initial purification step of immunoglobulin molecules like in the caprylic acid precipitation of non-IgG proteins, which is followed by cation exchange chromatography of hyperimmunized horse plasma. However, the later step is optional (WHO, 2018). The overall purpose is to maintain the purity and quality, to avoid adverse serum reactions post antivenom treatment (Raweerith and Ratanabanangkoon, 2003)

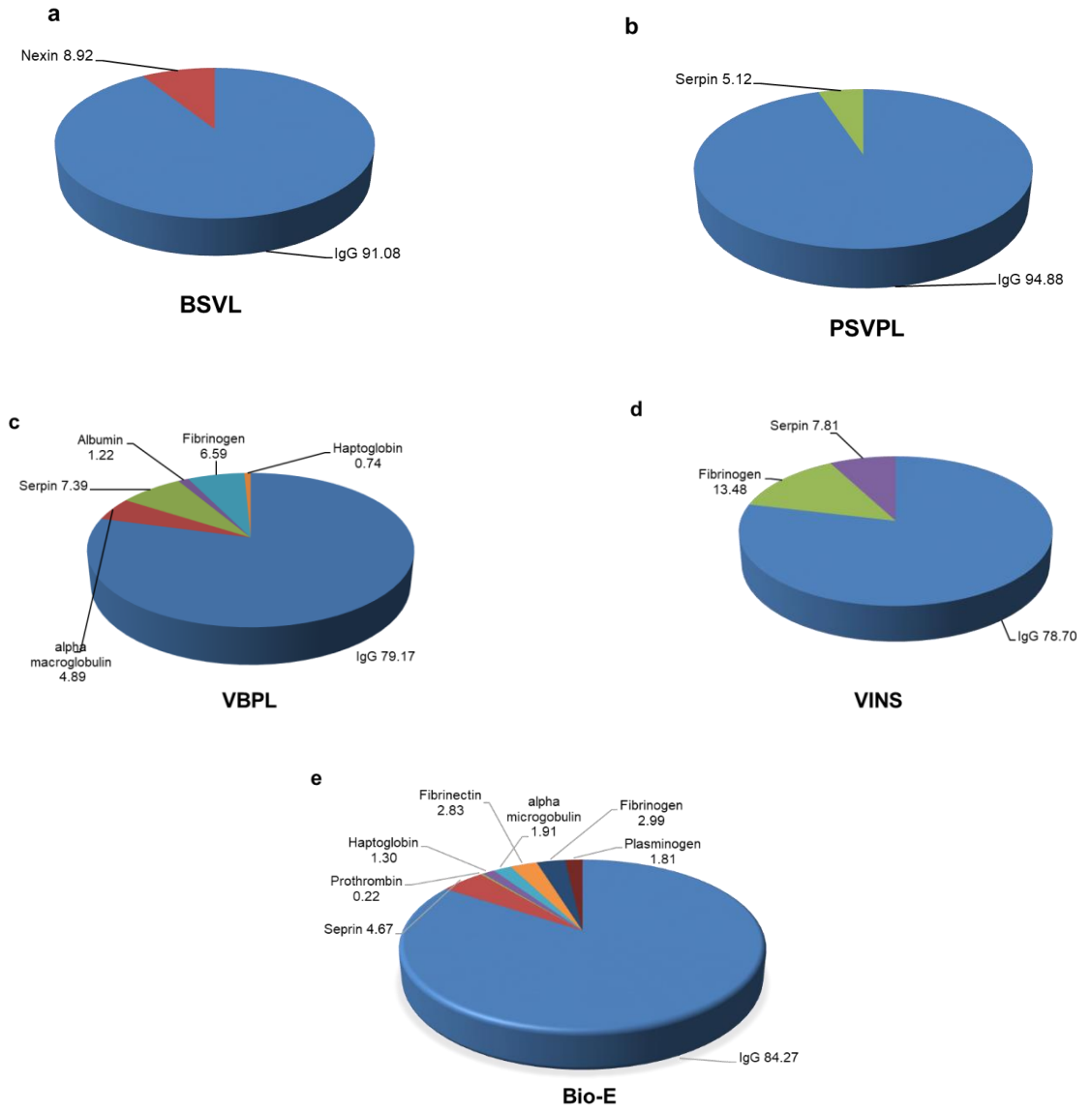
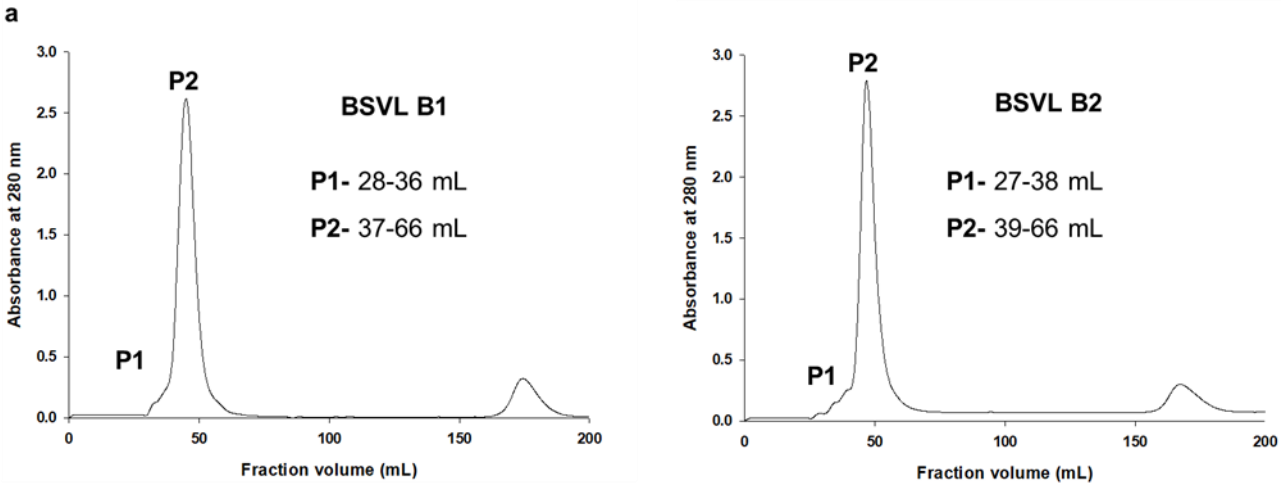


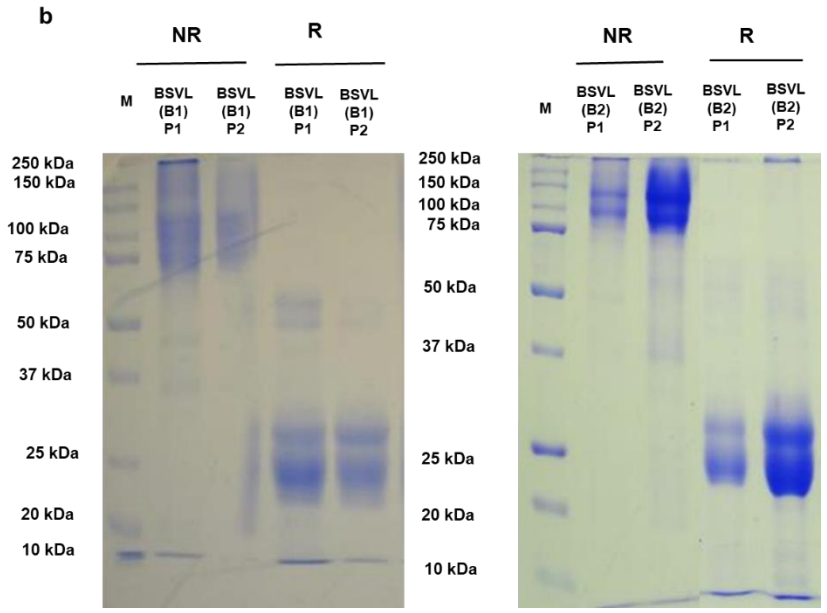
Fig 4. Determination of relative composition of PAVs manufactured in India by LC-MS/MS analysis. **a.** BSVL, **b.** PSVPL, **c.** VBPL, **d.** VINS, and **e.** Bio-E. The pie chart represents the relative occurrence of different protein families in PAVs.

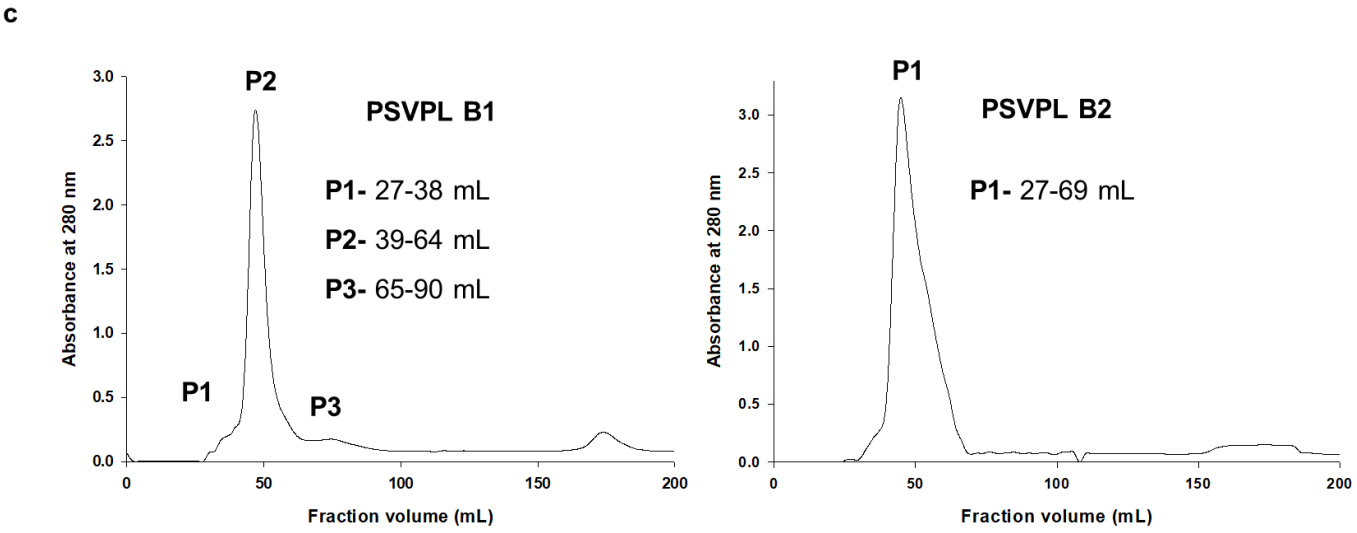
B1.3.4. Compositional analysis of PAVs by FPLC gel filtration chromatography followed by SDS-PAGE analysis of protein peaks:

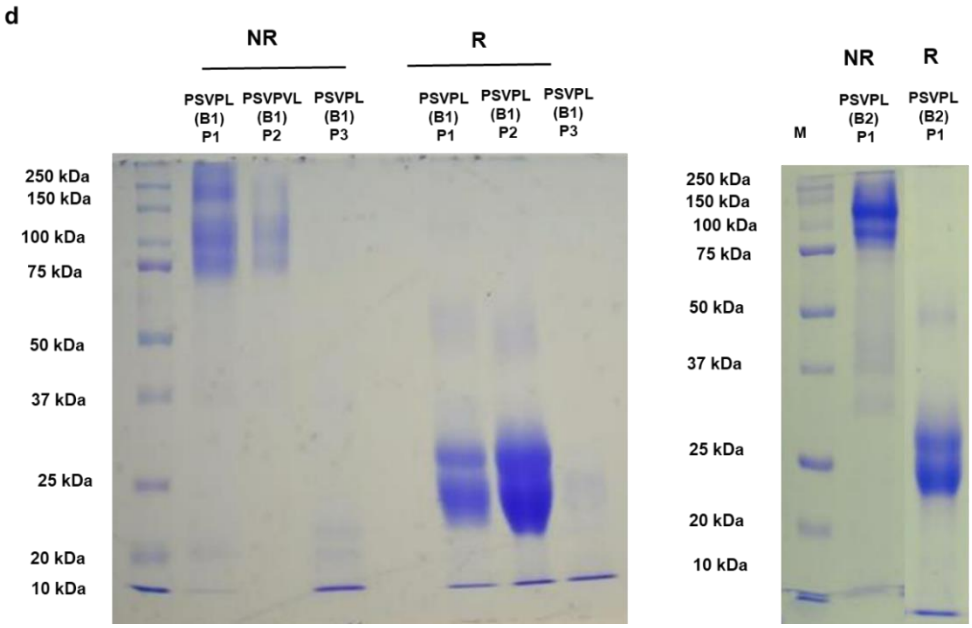
Fifty mg (dry weight) of antivenom samples were subjected to FPLC (AKTA Purifier 10, GE Healthcare, Sweden) gel-filtration chromatography on a Sephacryl S- 200 (16 x 600 mm) column along with the purified $F(ab')_2$ and horse IgG to compare with the elution pattern of antivenom samples (Patra et al., 2018).

To further ascertain the purity of the preparations, each PAV was subjected to FPLC gel filtration chromatography (Figs. 5a, c,e,g, i). The FPLC GF chromatogram resolved PAVs into one major peak (P1 for PSVPL batch 2 and P2 for the other PAVs) and two or one minor peak(s) (P1 and P3 for all the PAVs except PSVPL batch 2). In one case, the protein of the PAV (PSVPL batch 2) was eluted in a single peak (Fig. 5c). The protein content of the major GF peak was determined between 81 and 97% of the total venom protein. The elution times for the major peaks of each PAV were identical to the elution time of purified $F(ab')_2$ and horse IgG from the same column under identical experimental conditions (Patra et al., 2018). The peak P-1 of all PAVs except batch 2 of PSVPL was found to contain an $F(ab')_2$ dimer and aggregates, which was also supported by the SDS-PAGE analysis of this peak (Fig. 5b,d,f,h,j). The major protein peak 2 in all PAVs (except PSVPL batch 2) and peak 1 in PSVPL batch 2 showed a protein band at around 100 kDa and a band of apparent molecular weight 150 kDa by SDS-PADE analysis, suggesting the presence of a trace quantity of undigested IgG (Figs. 5b, d, f, h, j). Under reduced SDS-PAGE, the major peak of each PAV was separated into two distinct protein bands of molecular weight ~25 and ~35 kDa, suggesting that they were the heavy and light chains of IgG, respectively. A faint protein band of ~50 kDa was also observed by SDS-PAGE analysis indicating the presence of a heavy chain of the undigested IgG molecule (Figs. 5b, d, f, h, j).

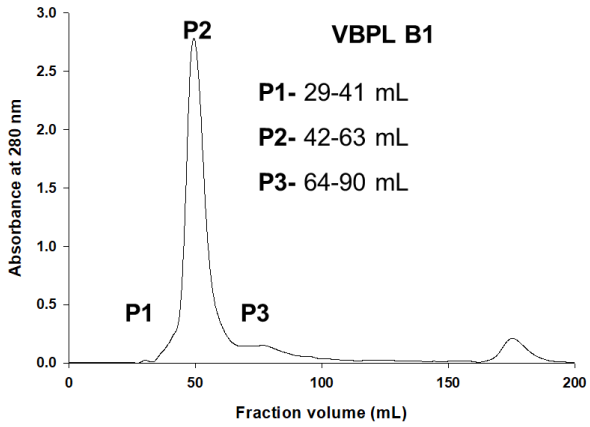


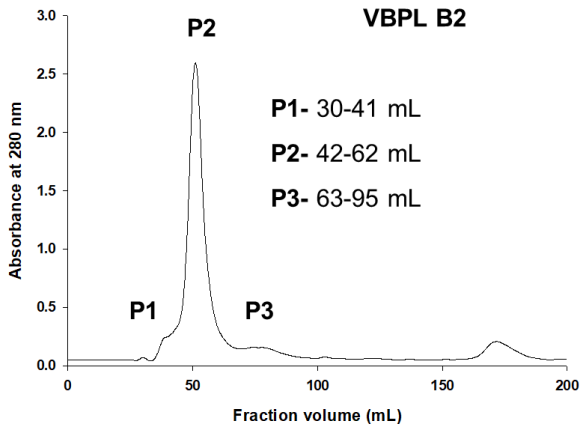

b
NR
R
M
BSVL (B1) P1
BSVL (B1) P2
BSVL (B1) P1
BSVL (B1) P2
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
10 kDa
NR
R
M
BSVL (B2) P1
BSVL (B2) P2
BSVL (B2) P1
BSVL (B2) P2
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
10 kDa


c
P2
PSVPL B1
P1- 27-38 mL
P2- 39-64 mL
P3- 65-90 mL
P1
P3
Absorbance at 280 nm
Fraction volume (mL)
P1
PSVPL B2
P1- 27-69 mL
Absorbance at 280 nm
Fraction volume (mL)

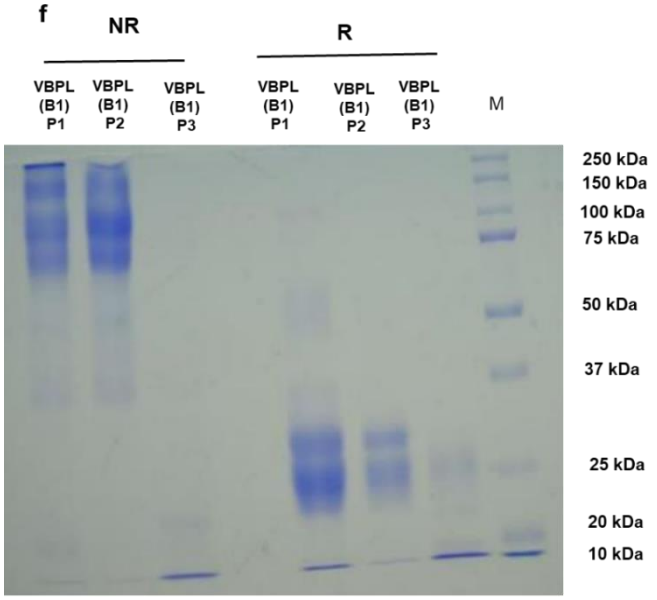

d
NR
R
PSVPL (B1) P1
PSVPVL (B1) P2
PSVPL (B1) P3
PSVPL (B1) P1
PSVPL (B1) P2
PSVPL (B1) P3
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
10 kDa
NR
R
M
PSVPL (B2) P1
PSVPL (B2) P1
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
10 kDa

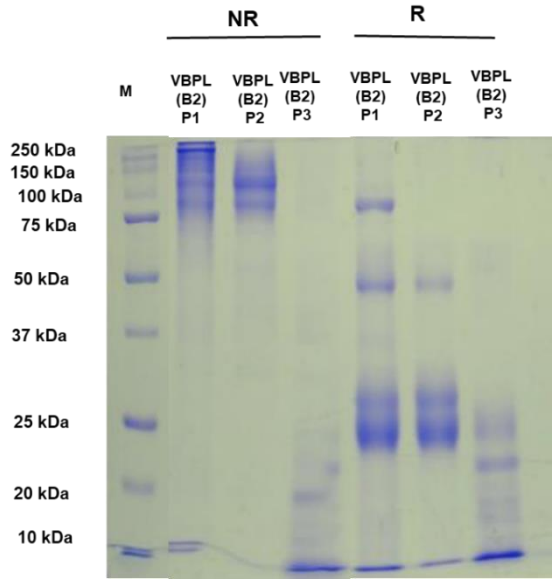
e


VBPL B1
P1- 29-41 mL
P2- 42-63 mL
P3- 64-90 mL
P1
P2
P3
Absorbance at 280 nm
Fraction volume (mL)

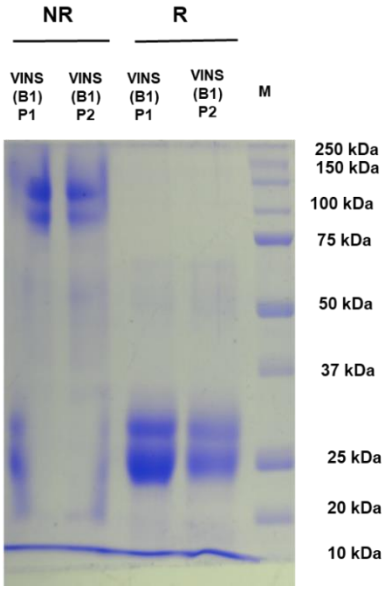

VBPL B2
P1- 30-41 mL
P2- 42-62 mL
P3- 63-95 mL
P1
P2
P3
Absorbance at 280 nm
Fraction volume (mL)

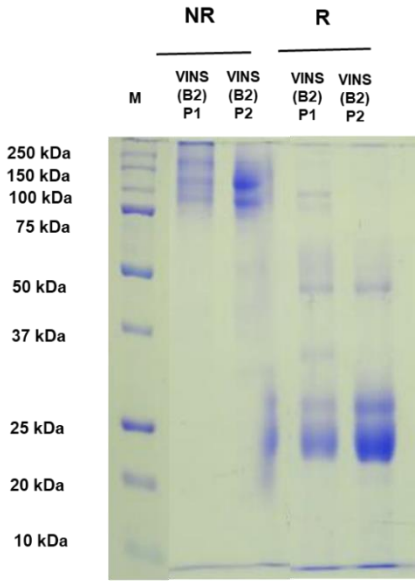
f


NR
R
VBPL (B1) P1
VBPL (B1) P2
VBPL (B1) P3
VBPL (B1) P1
VBPL (B1) P2
VBPL (B1) P3
M
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
10 kDa


NR
R
M
VBPL (B2) P1
VBPL (B2) P2
VBPL (B2) P3
VBPL (B2) P1
VBPL (B2) P2
VBPL (B2) P3
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
10 kDa

h


NR
R
VINS (B1) P1
VINS (B1) P2
VINS (B1) P1
VINS (B1) P2
M
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
10 kDa


NR
R
M
VINS (B2) P1
VINS (B2) P2
VINS (B2) P1
VINS (B2) P2
250 kDa
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10 kDa

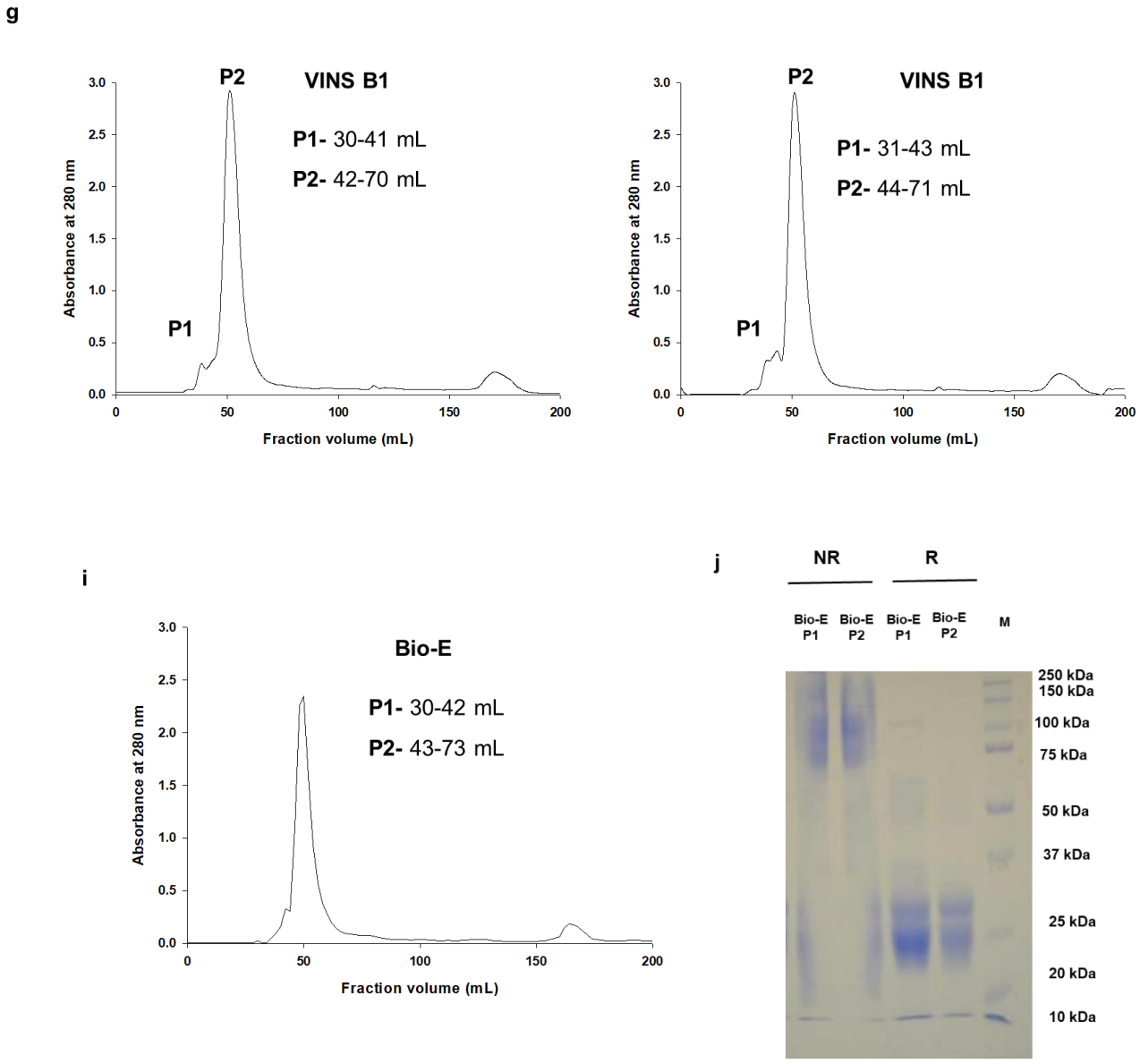
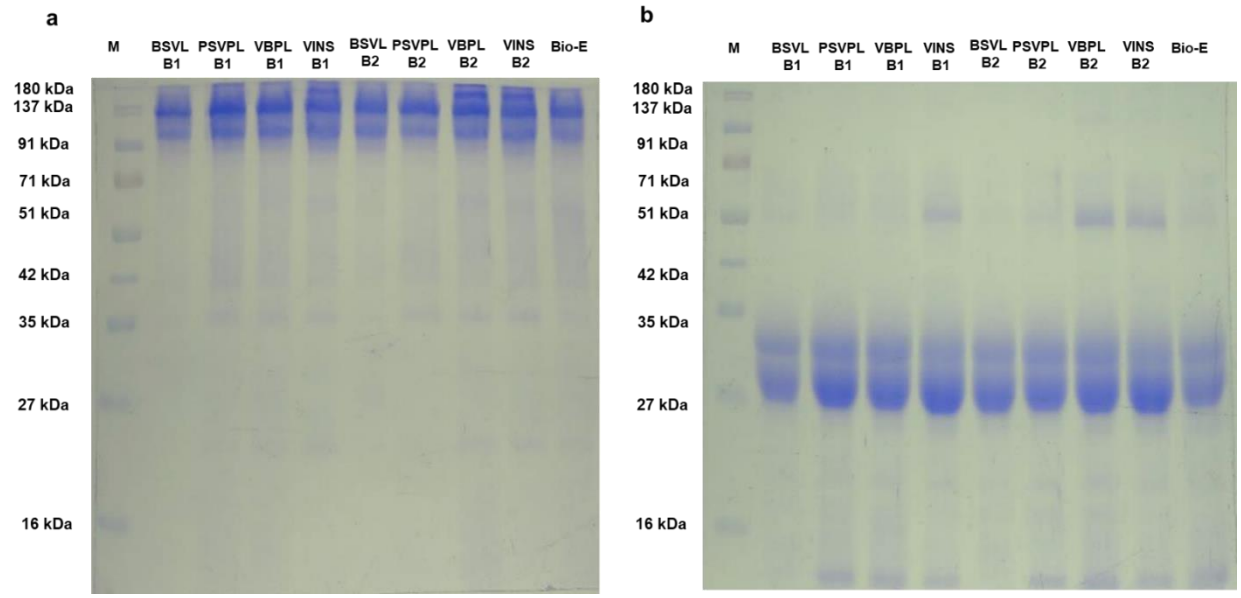


Fig. 5. FPLC size exclusion chromatography of PAVs and SDS-PAGE analysis of chromatographic peaks. **a, c, e, g and i** represent the gel filtration chromatogram of PAVs BSVL (batch 1and batch 2), PSVPL (batch 1and batch 2), VBPL (batch 1and batch 2), VINS (batch 1and batch 2) and Bio-E respectively. **b, d, f, h and j** represent the SDS-PAGE analysis of respective gel filtration peaks. B1 and 2: batch 1 and 2 respectively; P1-P3: peak 1 to peak 3 respectively.

B1.3.5. SDS-PAGE analysis of PAVs under reduced and non-reduced conditions

The composition of PAV samples was also determined by 12.5% SDS-PAGE analysis under reduced and non-reduced conditions.

Analysis of PAVs by SDS-PAGE (reduced) revealed two major protein bands at ~35 kDa and 20-25 kDa, representing the heavy and light chains, respectively, of partially digested IgG (Figs.6a-c). The densitometry analysis of IgG content from the SDS-PAGE profile of PAV is shown in Fig 6d. Nevertheless, a prominent protein band of ~50 kDa was observed in both batches of VINS and batch 2 of VBPL, representing partially undigested IgG in PAV preparation (Figs.6a-b). The SDS-PAGE analysis of PAV in non-reduced conditions showed a major protein band at around 110 KDa, which is similar to the molecular mass (100 kDa) of affinity-purified $F(ab')_2$ (Figs.6a, c). Nevertheless, the SDS-PAGE of PAV under non-reduced conditions also showed a faint protein band of ~150 kDa, indicating trace quantities of undigested IgG in the antivenom preparation (Figs.6a, c). The densitometry analyses of the SDS-PAGE bands revealed that batch 2 of VBPL (23.6%) and both batches of the VINS PAVs (16.8% and 27.3%, in batch 1 and batch 2, respectively) contained the highest amounts of IgG contamination (Fig. 5d) and the statistical analysis of data is shown in Table 11. The undigested IgG contamination in PAVs, other than those manufactured by VINS, range between 7 and 12% (Fig. 5d).



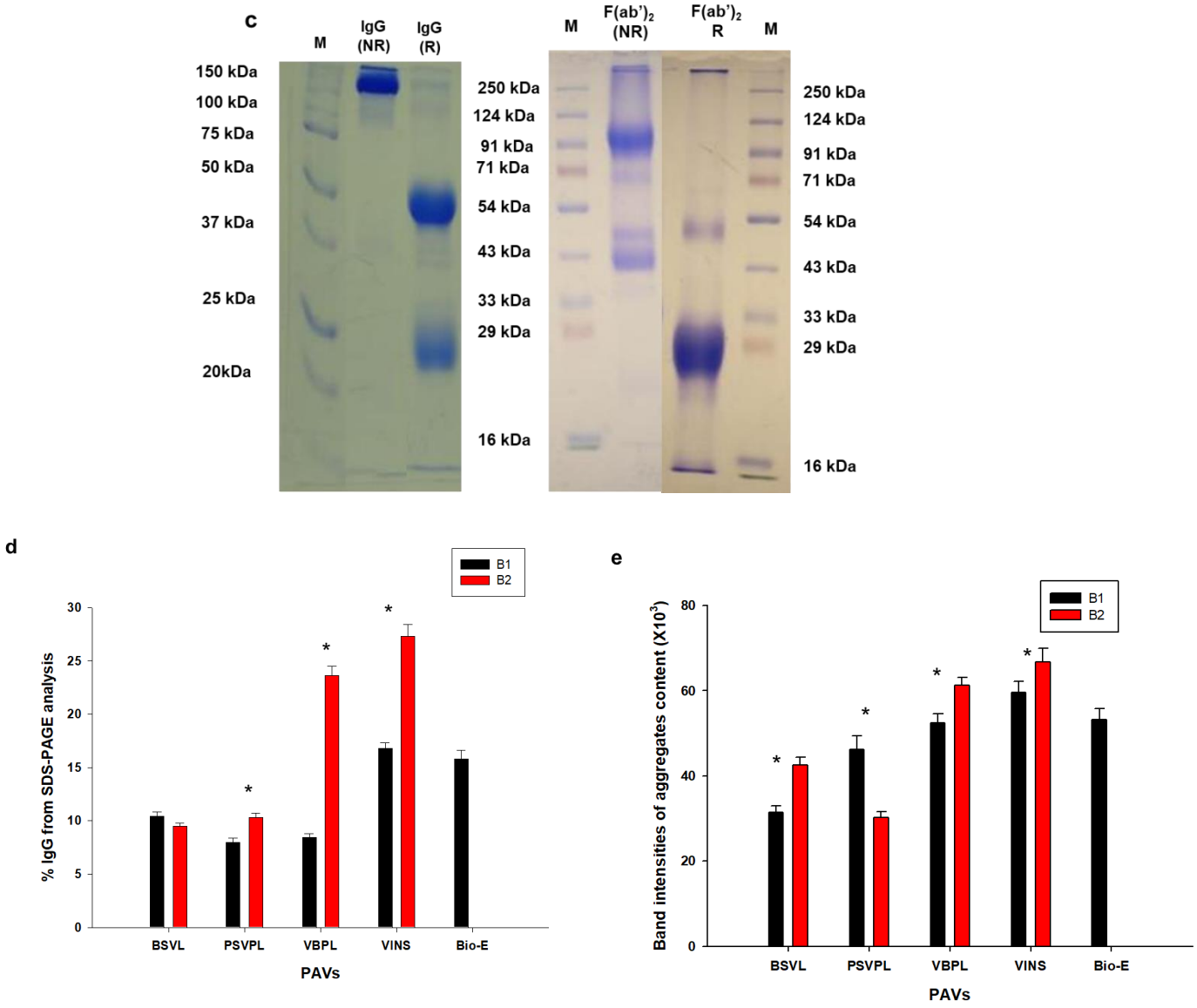


Fig. 6. The SDS-PAGE (12.5%) analysis of PAVs BSVL, PSVPL, VBPL and VINS in **a.** non-reduced condition, **b.** reduced condition, **c.** purified horse IgG and purified horse F(ab)2 molecule under non-reduced (NR) and reduced (R) condition. M represent protein molecular weight marker and B1 and B2 represent batch1 and batch2 of PAVs respectively. **d.** Percent composition of undigested IgG in each PAVs by densitometry scanning of SDS-PAGE. Values are mean ± SD for triplicate determinations. Significant differences within the batches of each antivenom manufacturer was considered as *p<0.05. Significance of difference among different antivenom manufacturers is shown in Table 11. **e.** Analysis of F(ab')2 aggregates in the PAVs (B1 and B2) by SDS-PAGE analysis. Values are mean ± SD for triplicate determinations. Significance of differences between the two batches of PAV produced by same

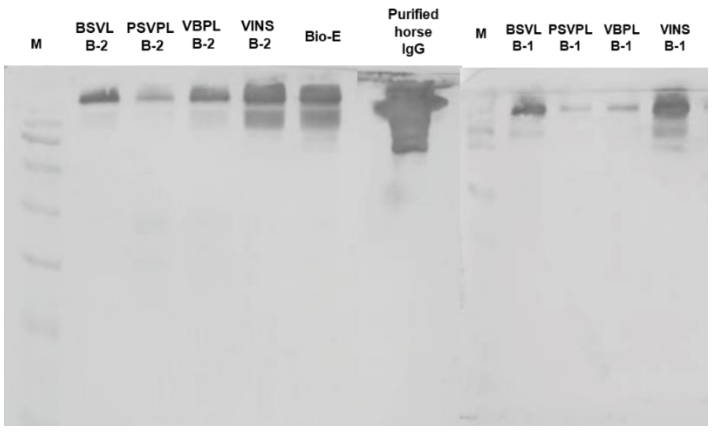
manufacturer, *p<0.05. Significance of differences between the two batches of PAV produced by the same company, *p<0.05. The detail statistical analysis of data is shown in Table 11).

B1.3.6. Determination of Fc content of IgG by immunoblotting analysis

Undigested IgG retains its Fc portion; therefore, quantitative determination of Fc would provide information on proportion of undigested IgG in F(ab')2 antivenom. To determine the occurrence of incompletely digested IgG, the Fc content in each batch of PAVs was determined by immunoblot analysis using anti-horse IgG Fc-specific antibody conjugated with HRP (Sigma Aldrich, USA) (Patra et al., 2017; Patra et al., 2018). The densitometry analysis was done with Image Quant TL software (GE Healthcare, Sweden). The percent content of Fc in the purified horse IgG was considered as 100% and other values were measured relative to that.

The Fc content in undigested/partially digested IgG in PAV preparations was also quantitated by ELISA using anti-IgG-Fc-specific antibody. The ELISA study confirmed the presence of different proportions of partially digested/ undigested IgG in PAV (Figs. 7c). The Fc content determined by the immunoblotting method is well corroborated with the ELISA data (Figs. 7c) and percent IgG content determined by SDS-PAGE (reduced) analysis of PAVs (Fig. 6d). Because the Fc portion of the undigested IgG is responsible for early adverse serum reactions in antivenom-treated patients (Cardoso et al., 1993; Isbister et al., 2012), the pepsin digestion protocol should further be standardized or be carefully followed to avoid incomplete/partial digestion of IgG. Moreover, it may be suggested that affinity purification of $F(ab')_2$ may be an alternative way for improving the quality of PAV (Lalloo and Theakston, 2003), though the process may not be economical for the developing countries.

a



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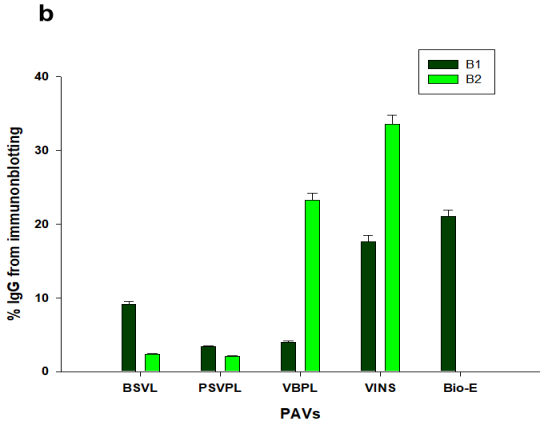

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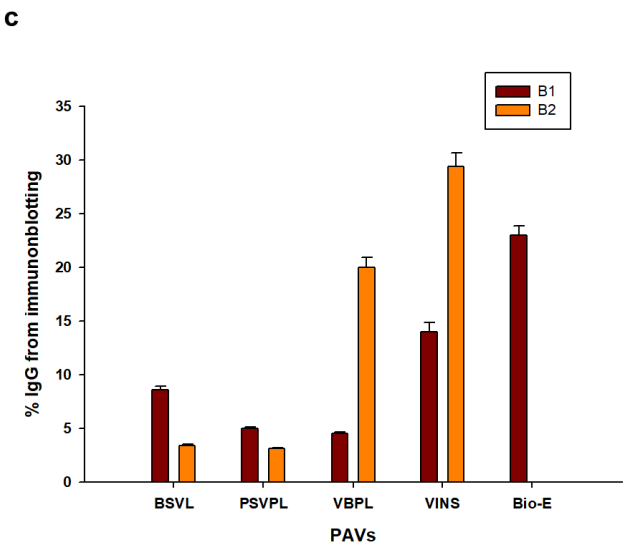

Fig.7. a Immuno-detection of Fc content of the antivenom by using anti horse IgG HRP conjugated Fc region- specific antibody. M represent protein molecular weight marker and B1 and B2 represent batch1 and batch2 of PAVs respectively. **b.** Densitometry analyses of Fc content from blot intensities. Significance of manufacturer was considered as *p<0.05. The detail statistical analysis of data is shown in Table 11. **c.** Determination of Fc content by ELISA using anti-horse IgG HRP conjugated Fc region-specific antibody. Values are mean ± SD with triplicate determinations.

B1.3.7. Determination of IgA and IgE contamination, if any, in PAV by ELISA and immunoblot analysis:

The possibility of co-separation of other immunoglobulin molecules, such as IgA and/or IgE along with the IgG molecule from hyper-immune horse serum may not be ruled out (Patra et al., 2018). IgA and IgE contamination in the PAVs, if any, was determined by ELISA and immunoblot analysis, as described previously (Patra et al., 2018). Contamination of IgE in the antivenom preparations may induce a hypersensitivity reaction and cause anaphylactic shock (Williams, 2007), though adverse reaction(s) to IgA have not been demonstrated. The results from ELISA and the immunoblot studies reveal that none of the PAVs tested were contaminated with IgE from horse plasma (data not shown), but they contained IgA molecules (Figs. 8a-c).

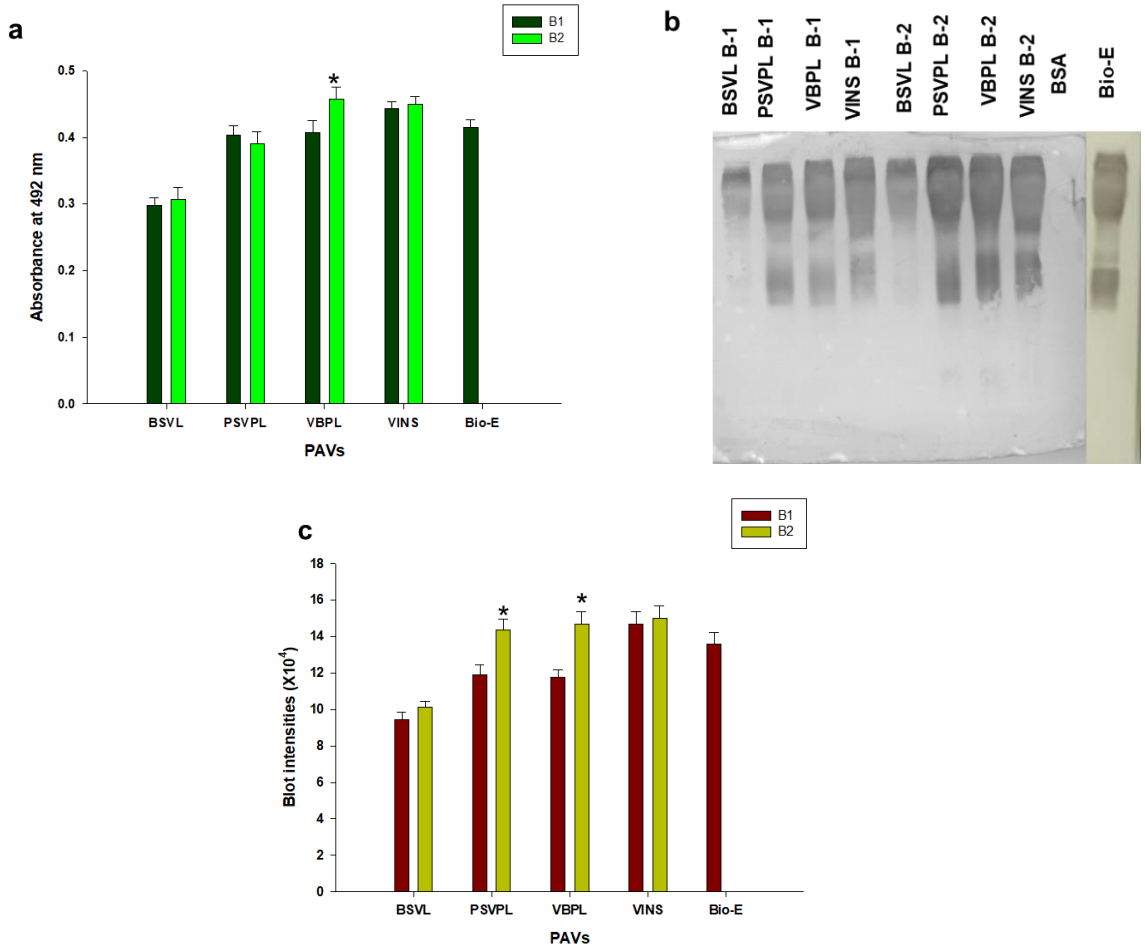

Fig. 8. a. Determination of immunological cross-reactivity of PAVs (batch 1 and batch 2) against anti-horse IgA antibodies (HRP-conjugated) by ELISA. Values are mean ± SD with triplicate determinations. **b.** Immuno-blot analysis of IgA contamination in PAVs batch 1 (B-1) and batch 2 (B-2). The immuno blots were detected by HRP conjugated anti-horse IgA antibody. The BSA was used as a negative control. **c.** Band intensity of IgA in PAVs detected by densitometry analysis of immunoblotting. Significance of difference between the two batches of PAV, *p<0.05. The detail statistical analysis of data is shown in Table 11.

B1.3.8. Safety profiles of PAV: Determination sterility, cell cytotoxicity and endotoxin level in PAVs:

The sterility of the PAV was tested according to WHO guidelines (WHO, 2018) and found to be free of microbial contamination. The PAV preparation in addition of containing active substance [$F(ab')_2$] also contains small amounts of other plasma proteins, preservatives, stabilizers and IgG aggregates. Depending on the severity of envenomation, repeated doses of PAV would be administered to the

patient over a 2-3 day period or may be for a longer duration (Mukherjee et al., 2020). Therefore, in order to explore the adverse effect of PAV, if any, on the tissues and erythrocytes, we assessed the *in vitro* cell cytotoxicity of PAVs (20 µg/mL) post 72 h of treatment on mammalian cells. None of the PAVs showed cytotoxicity against mammalian cells (Fig. 9a-b) or direct haemolytic activity against mammalian erythrocytes (data not shown).

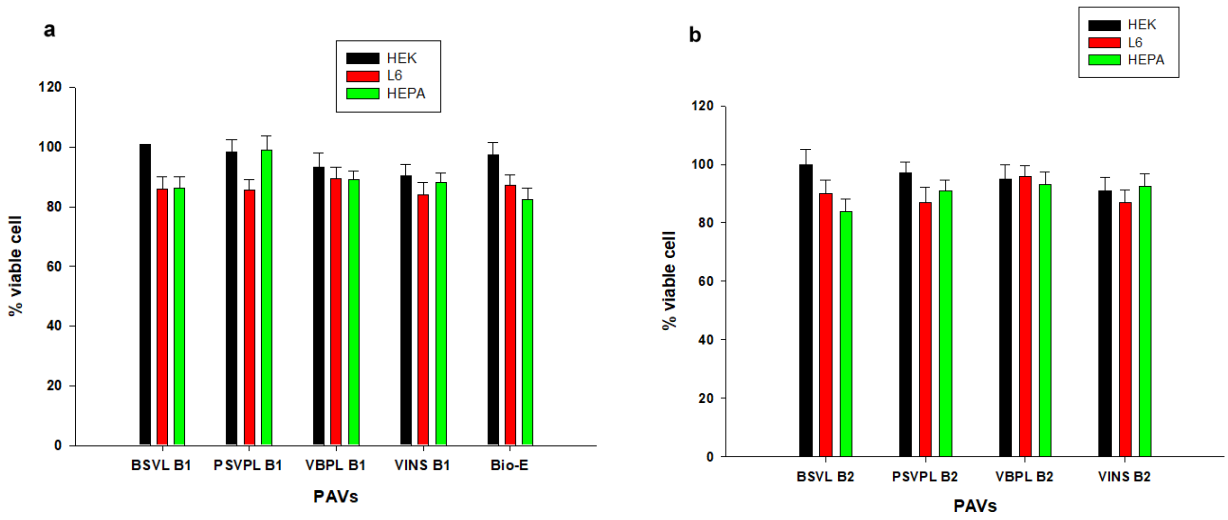

Fig. 9. Assessment of cell cytotoxicity of PAVs, if any, against human embryonic kidney (HEK293T), mouse hepatocyte (Hepa 1-6) and differentiated L6 myotubes cells. The **a,** and **b** represents batch B1 and B2, respectively. The cytotoxicity was assessed post 72 h of treatment. Values are mean ± SD of triplicate determinations. Significance of difference with respect to control, $p>0.05$.

The threshold level of endotoxin in antivenom for doses 20 mL to 120 mL is 2.9-17.5 EU/mL and above that limit, pyrogenic reactions can develop in PAV-treated patients (Solano et al., 2015). PAVs were screened for possible bacterial endotoxin contamination using a Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, USA), following the manufacturer's protocol (Patra et al., 2018). Depending on the species of snake and severity of envenomation, approximately 20-120 mL intravenous administration of PAV is required and the maximum level of endotoxin should range between 2.9 and 17.5 EU/mL(Solano et al., 2015). The quantity of endotoxin in all of the tested batches of PAVs was found to be in the range of 0.5 to 1.6 EU/mL, which is far below the threshold level, suggesting that none of the PAVs were contaminated with bacterial endotoxin(s) (Fig. 10, the statistical analysis of data is shown in Table 11).

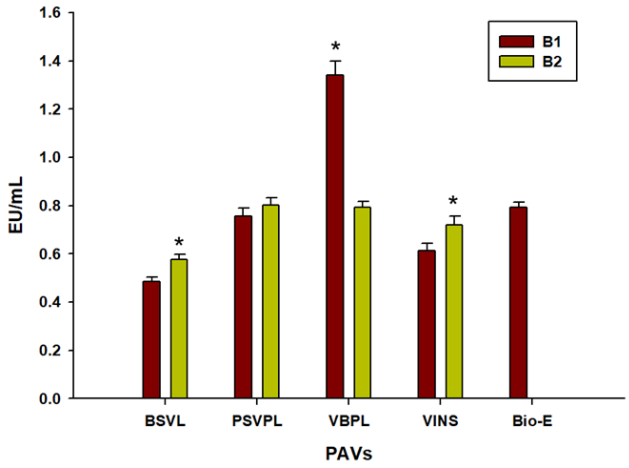

Fig. 10. Determination of endotoxin contamination, if any, in different batches of PAV. Values are mean ± SD of triplicate determinations. Significance of differences (*p<0.05) among PAV produced by different manufacturers is shown in Table 11.

B1.3.9. Assessment of complement activation by PAV by classical and alternative pathway

The Fc part of the undigested IgG in PAVs may induce complement activation (García et al., 2002). Recent findings; however, have shown that $F(ab')_2$ aggregates (Eursakun et al., 2012), the hinge portion of IgG (Squaiella-Baptistao et al., 2014), and a high content of preservatives (cresol/phenol) (García et al., 2002) are likely responsible for complement activation.

Complement activation induced by PAVs via classical and alternative pathways was determined by the method originally described by Pidde-Queiroz et al., (2010) (Pidde-Queiroz et al., 2010). Complement activity was measured by CH50 (total haemolytic complement) or AP50 value (alternative pathway complement measurement) as described by Squaiella-Baptista˜o et al., (2014). The CH50 (classical pathway) and AP50 (alternative pathway) was calculated by comparing the activity of NHS incubated with normal saline (100% activity) (Squaiella-Baptistao et al., 2014). The tested PAVs showed moderate level of complement activation (Fig. 11a-b). This finding is well correlated with the occurrence of higher proportions of $F(ab')_2$ aggregates and partially undigested IgG (containing the Fc region) in the PAVs that show the higher complement activation (Figs. 6c,e; 7a-b). Complement activation by PAVs may also result in the release of anaphylotoxin that causes hypersensitivity reactions in

antivenom-treated patients (Abbas et al., 2002) suggesting that quality assurance of PAV may reduce complement activation and other adverse reactions.

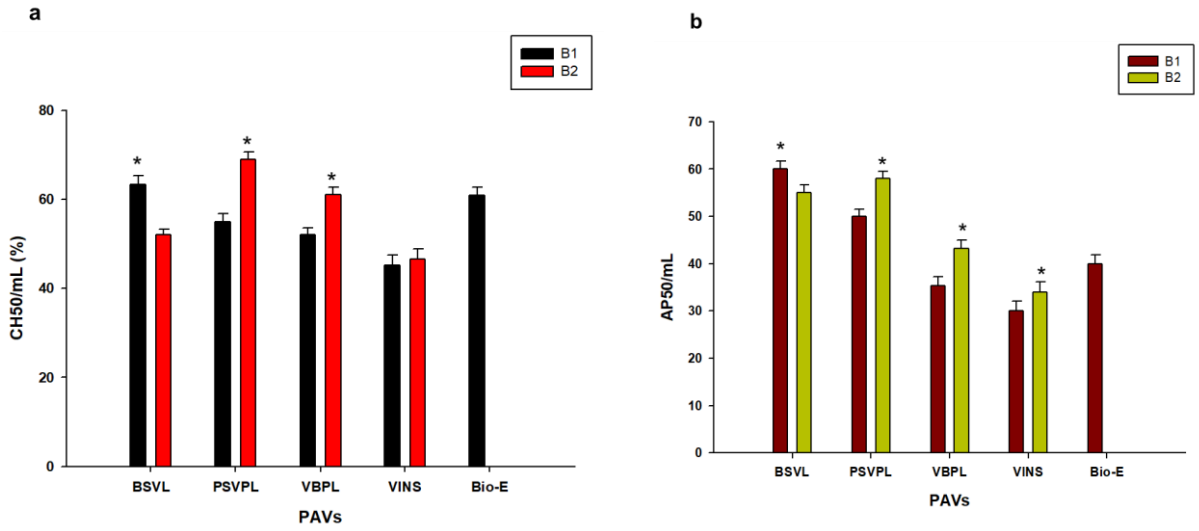

Fig 11. Determination of the complement activation pathways of PAVs (B1 and B2) **a.** Classical pathway. **b.** Alternative pathway. The experimental details are described in the text. Values are mean ± SD for triplicate determinations. Significance of difference of complement activation properties between the two batches of PAV, *p<0.05. The detail statistical analysis of data is shown in Table 11.

B1.3.10. Determination of cresol (preservative) level in PAVs

The percentage of cresol in PAVs was determined by reversed-phase ultra-high performance liquid chromatography (RP-UHPLC) as described by Patra et al., (2018). PAVs are formulated with the preservative cresol for long-term storage. Nevertheless, an excessive amount of cresol (>3.5%) in the antivenom preparation (WHO, 2018) can lead to complement activation and a hypersensitive reaction in antivenom-treated patients(García et al., 2002; Rane et al., 2011). The PAVs in the present study contained 0.04-0.1% m-cresol, indicating that they followed the recommendation of the WHO (Fig. 12).

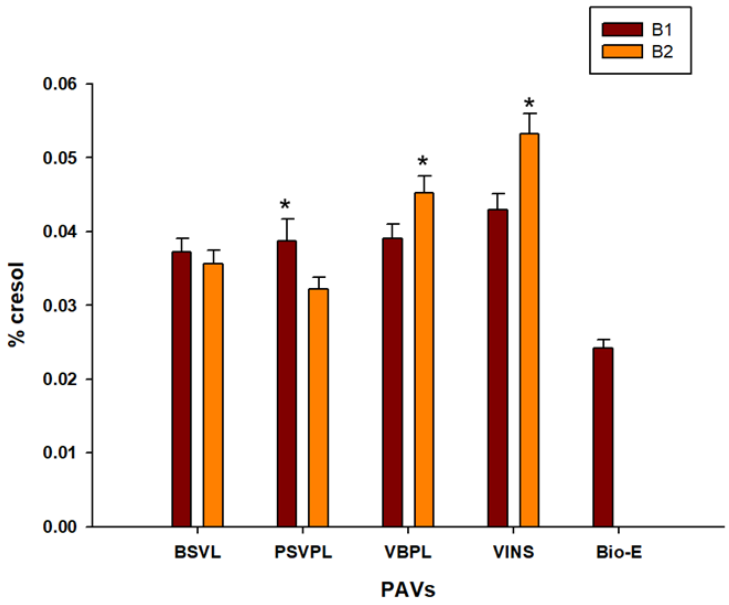

Fig 12. Determination of *m*-cresol content in different batches of PAVs. Significance of difference between the two batches of PAV, *p<0.05. The detail statistical analysis of data is shown in Table 11.

Table 11: The statistical analysis of Figures 6d-e, 6b, 8a, 8c, 9, 10a-b, and 11. The Significance of differences within the commercial antivenom were considered as *p < 0.05, **p < 0.01, **p < 0.001. NS: not significant (p>0.05).

	Significance of difference								
Comparison	**Fig. 6d**	**Fig. 6e**	**Fig. 7b**	**Fig. 8a**	**Fig. 8c**	**Fig. 11a**	**Fig. 11b**	**Fig. 10**	**Fig. 12**
BSVL B1 vs PSVPL B1	**	***	***	***	**	NS	NS	***	NS
BSVL B1 vs VBPL B1	*	***	***	***	**	NS	NS	***	NS
BSVL B1 vs VINS B1	***	***	***	***	***	***	***	***	NS
BSVL B1 vs Bio-E	***	***	***	***	***	NS	***	***	***
BSVL B1 vs BSVL B2	NS	***	***	NS	NS	NS	NS	**	NS
BSVL B1 vs PSVPL B2	NS	NS	***	***	***	NS	NS	***	NS
BSVL B1 vs VBPL B2	***	***	***	***	***	NS	*	***	**
BSVL B1 vs VINS B2	***	***	***	***	***	***	***	***	***
PSVPL B1 vs VBPL B1	NS	NS	NS	NS	NS	NS	NS	***	NS
PSVPL B1 vs VINS B1	***	***	***	*	***	***	***	***	NS
PSVPL B1 vs Bio-E	***	*	***	NS	NS	NS	**	NS	***

PSVPL B1 vs BSVL B2	NS	NS	NS	***	*	NS	NS	***	NS
PSVPL B1 vs PSVPL B2	**	***	NS	NS	**	NS	NS	NS	*
PSVPL B1 vs VBPL B2	***	***	***	**	***	*	NS	NS	NS
PSVPL B1 vs VINS B2	***	***	***	*	***	***	***	NS	***
VBPL B1 vs VINS B1	***	**	***	NS	***	***	***	***	NS
VBPL B1 vs Bio-E	***	NS	***	NS	*	NS	*	***	***
VBPL B1 vs BSVL B2	NS	***	NS	***	NS	NS	NS	***	NS
VBPL B1 vs PSVPL B2	NS	***	NS	NS	***	**	NS	***	*
VBPL B1 vs VBPL B2	***	**	***	**	***	NS	NS	***	NS
VBPL B1 vs VINS B2	***	***	***	*	***	***	***	***	***
VINS B1 vs Bio-E	NS	*	***	NS	NS	***	*	***	***
VINS B1 vs BSVL B2	***	***	***	***	***	***	***	NS	*
VINS B1 vs PSVPL B2	***	***	***	**	NS	***	***	***	***
VINS B1 vs VBPL B2	***	NS	***	NS	NS	***	***	***	NS
VINS B1 vs VINS B2	***	**	***	NS	NS	NS	NS	**	***
Bio-E vs BSVL B2	***	***	***	***	***	NS	*	***	***
Bio-E vs PSVPL B2	***	***	***	NS	NS	NS	***	NS	**
Bio-E vs VBPL B2	***	**	*	*	NS	NS	NS	NS	***
Bio-E vs VINS B2	***	***	***	NS	NS	***	NS	NS	***
BSVL B2 vs PSVPL B2	NS	***	NS	***	***	NS	NS	***	NS
BSVL B2 vs VBPL B2	***	***	***	***	***	**	NS	***	***
BSVL B2 vs VINS B2	***	***	***	***	***	***	***	***	***
PSVPL B2 vs VBPL B2	***	***	***	***	NS	***	*	NS	***
PSVPL B2 vs VINS B2	***	***	***	**	NS	***	***	NS	***
VBPL B2 vs VINS B2	***	NS	***	NS	NS	***	**	NS	**

B1.3.11. Determination of venom-specific antibodies in PAV in by spectrofluorometric titration

The binding affinity of PAV towards snake venom samples obtained from different geographical locations in India was determined by intrinsic fluorescence analysis as described previously (Dutta et al., 2019; Kalita et al., 2019) with slight modifications. Briefly, increasing concentrations (final concentration from 0.01 mg/mL to 2.5 mg/mL) of PAV (the two batches were mixed at 1: 1 ratio according to protein content) were added into the wells of a Nunc™ F96 MicroWell™ Black Polystyrene

Plate (Thermo Fisher Scientific, Denmark) containing 10 µL of 1 mg/ mL of venom sample and incubated for 3 min at room temperature. The venom and antivenom ratio (w/w) was maintained from 1:1 to 1:256. The reaction mixture (venom-antivenom conjugate) was excited at 280 nm and the emission spectrum was measured between 300 and 500 nm. The slit length was maintained at 10 nm. As a control the fluorescence spectrum of individual proteins was also determined and compared with relative intensities of the spectra of venom-PAV complex. The binding experiments were done in triplicate and the average of 5 scans was used for the construction of binding curve.

The dissociation constant (K_d value) was determined by plotting the change in λmax against the concentration of the antivenom (mg protein/mL) using one site-specific binding model of the interactions in the GraphPad Prism 5.0 software (Dutta et al., 2019; Kalita et al., 2019). The PAV contains varying proportion of $F(ab')_2$ and IgG; therefore, the values were expressed as mg PAV protein/mL. From the one site binding curve of PAV-venom interaction, the amount of PAV that shows saturation in binding with a fixed amount of test venom sample (when no further increase in fluorescence signal of venom – antivenom mixture was observed) was determined. The total amount of immunoglobulin [IgG and/or $F(ab')_2$] in a vial was calculated from the amount of immunoglobulin molecules present in per g protein (determined by LC/MS-MS analysis data). The venom-specific antibodies in PAV (w/w) was determined by using the following formulas

$$A.\,Total\ venom-specfic\ antibodies\ in\ a\ vial\ (mg)$$

$$= \frac{Amount\ of\ venom\ used\ in\ spectrofluorometric\ titration(mg)}{Amount\ of\ PAV\ in\ which\ shows\ \ saturation\ with\ the\ venom\ (mg)}$$

$$\times\ Total\ amount\ of\ immunoglobulin\ content\ [IgG\ and/or\ F(ab')2]\ \ in\ a\ vial\ (mg)$$

$$B.\,Percentage\ of\ venom-specfic\ antibodies\ in\ a\ vial$$

$$= \frac{Total\ venom-specific\ antibodies\ in\ a\ vial\ (\mathrm{mg})}{Total\ immunoglobulin\ content\ [IgG\ and/or\ F(ab')2]\ in\ a\ vial\ (\mathrm{mg})} \times 100$$

In this study, we have determined the venom-antivenom interaction by spectrofluorometric titration curve. More is the venom-specific antibody in a PAV, higher will be the spectrofluorometric signal of venom-PAV interaction. Increasing the concentration of PAV results in gradual increase in the fluorescence signal and when all the binding sites of the PAV were occupied by venom, no further increase in fluorescence signal was observed indicating saturation is achieved. Therefore, this technique was used to determine the binding affinity of commercial PAV to the venoms of the 'Big four'

and *N. kaouthia* snakes from different geographical regions of the country and from the saturation of binding, venom-specific antibodies in PAVs were determined.

The K_d value determines the strength of the bio-molecular interaction of PAV to a particular component of venom; the smaller is the K_d value, the higher would the binding affinity of venom with PAV. Therefore, K_d value determines the quantitative affinity of a given antivenom (antibody) against venom antigens. The K_d value of interaction between PAV and venoms under study was determined in mg protein content of PAV /mL [which is equivalent to micromolar concentration of $F(ab')_2$ and/or IgG] suggests the high affinity of antibodies against their venom antigens. Therefore, this result ascertains the presence of a reasonably good quantity of venom-specific antibodies in commercial PAV. The K_d values for all of the PAVs were found to be lower for ECV and DRV (Viperidae) samples, compared to those for NNV, BCV, and NKV (Elapidae) samples (Table 12). Thus, the PAVs had greater binding efficiencies with the venoms from snakes of the Viperidae, compared to the Elapidae, which reinforces presence of a higher proportion of Viperidae venom-specific antibodies compared to Elapidae venom-specific antibodies in the PAVs, as shown below.

In this study, the presence of each species of snake venom-specific antibodies in commercial PAVs was determined by spectrofluorometric titration method. The PAVs contained different proportions of antibodies against different species of the 'Big Four' snake venoms, and also against *N. kaouthia* venom (Fig. 12, and the statistical analysis of data is shown in Table 13). Although later venom was not used in the immunogen mixture for raising polyclonal antibodies in horses; nevertheless, because of antigenic epitope sharing among several toxins of Indian monocled cobra venom with Indian spectacled cobra (homologous species) or Indian common krait (heterologous species) venoms, the *N. kaouthia* venom was also immune-recognized and partially neutralized by PAV. The highest proportion of antibodies was found against ECV (13.3 -16.9%), followed by DRV (9.9-15.3%), NNV (7.1-10.8%), and finally, BCV (5.5-6.9%) and NKV samples (6.8-7.8%) (Fig. 13, Table 13).

Table 12: Determination of K_d values (in mg protein/mL) for interaction between specific venom and PAV by spectrofluorometric analysis. The experiment is described in the text. The values are average of triplicate determinations.

PAVs manufacturing companies	Venom samples								
	DRV WI	DRV EI	DRV SI	NNV WI	NNV EI	NNV SI	ECV SI	BCV SI	NKV EI
	Dissociation constant (K_d value) for venom-PAV interaction (mg protein/mL)								
BSVL	0.4	0.4	0.3	0.5	0.5	0.5	0.3	0.7	0.6
PSVPL	0.4	0.4	0.4	0.5	0.5	0.5	0.3	0.7	0.6
VBPL	0.5	0.5	0.4	0.6	0.6	0.6	0.3	0.8	0.7
VINS	0.4	0.5	0.4	0.6	0.6	0.6	0.4	0.9	0.7
Bio-E	0.5	0.5	0.4	0.6	0.7	0.6	0.3	0.9	0.7

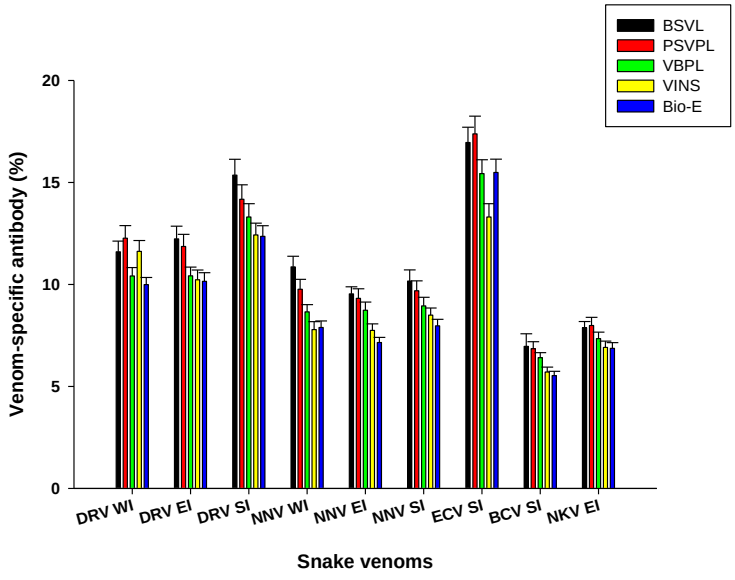

Fig 13. Determination of percentage of venoms-specific antibodies in Indian PAVs against the 'Big Four' snakes and *N. kaouthia* by spectrofluorometric titration. The Values are mean ± SD of triplicate determinations. Significance of differences ($p<0.05$) in containing the venom-specific antibodies in PAVs are shown in Table 13.

Table 13: The statistical analysis of Figure 11. The Significance of differences of venom specific antibodies in PAVs, *p < 0.05, NS: not significant (p>0.05).

Venom samples	**Significance of difference (p value)**									
	BSVL vs PSVPL	**BSVL vs VBPL**	**BSVL vs VINS**	**BSVL vs Bio-E**	**PSVPL vs VBPL**	**PSVPL vs VINS**	**PSVPL vs Bio-E**	**VBPL vs VINS**	**VBPL vs Bio-E**	**VINS vs Bio-E**
DRV WI	NS	*	*	*	*	NS	*	NS	*	NS
DRV EI	NS	NS	*	*	*	*	*	*	*	NS
DRV SI	NS	*	*	*	NS	*	*	*	*	NS
NNV WI	NS	*	*	*	NS	*	*	*	*	NS
NNV EI	NS	NS	*	*	*	*	*	*	*	NS
NNV SI	NS	*	*	*	NS	*	*	NS	*	*
ECV SI	NS	*	*	*	NS	*	*	*	*	NS
BCV SI	NS	NS	NS	*	NS	NS	*	NS	NS	NS
NKV EI	NS	NS	*	*	NS	*	*	NS	NS	NS

Conclusion: A Road Map for improvement of snakebite envenomation in India and in ASEAN countries:

Snakebite is a severe problem in India and ASEAN countries, yet measures to eradicate this neglected tropical diseases are rendered conspicuous by their absence. Controlling the incident of snakebite in the countries and improving the treatment of snakebite is a challenging task; nevertheless, it is an achievable goal. At this juncture, it is extremely important to formulate a roadmap and lay down the guidelines for the prevention and improvement of hospital management of snakebite in the Southern Asian countries, which have experienced the highest incidence of snakebite in the world. A blueprint covering both short term and long term goals, achievable within 5 and 10 years, respectively, pertaining to the improvement of therapy, augmentation of clinical research on snakebite, emphasis on social awareness to prevent snakebite and best practices for snakebite treatment with the existing facility and technology in a particular locality, is presented in Table 14.

Table 14: A summary of the blueprint for the improvement of treatment and prevention of snakebite in South Asian and ASEAN countries.

1	Regional epidemiological surveys on snakebite, identification of major venomous species of snakes in different locals of the country, and formulation of snakebite data governess policy
2	Specialized training of medical and paramedical staff, including clinicians, on snakebite treatment
3	Development of snake venom detection kits
4	Improvement of efficacy, quality control and quality assurance, and rational distribution of commercial antivenoms
5	Encouraging research on new methods of antivenoms production and emphasis on developing alternative treatment(s) for snakebite
6	Production of country-specific antivenoms and moving beyond ‘Big Four’ venomous snake antivenoms
7	Establishment of regional centers for collection and testing of purity of venoms
8	Adequate funding for basic and clinical research on snake venom

9	Legislative reforms, increasing regional and international cooperation in snakebite therapy
10	Increasing social awareness on the prevention of snakebite and following best practices for snakebite treatment

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Action taken report against the approved objectives

No. of Objectives	Objectives	Achieved or not.	Remarks
1	To study the epidemiology of snakebite in some selected districts of Assam, NE India, in Malaysia and in Vietnam.	Yes	
2	To investigate the pathophysiology and clinical correlation of snakebite envenomation as well as the effectiveness of snakebite management at district hospitals and tertiary care centers in some selected districts of Assam, NE India, Malaysia, and Vietnam	Yes	
3	To develop the standardization of laboratory tests for assessment of efficacy and safety of commercial polyvalent/monovalent antivenom manufactured in India and ASEAN countries.	Yes	

B3. Laboratory Exchange Visit: (Reports are submitted after each visit)

1. Prof. A.K. Mukherjee (Indian PI) visited Department of Pharmacology, University of Malaya, Malaysia (2018).
2. Prof. A.K. Mukherjee (Indian PI) visited Department of Toxicology and Radiology, Vietnam Military Medical University (VMMU), Hanoi, Vietnam (2018).
3. Mr. Aparup Patra (Project SRF) visited Department of Pharmacology, University of Malaya, Malaysia (2019)
4. Dr. L.V. Dong (Vietnamese Collaborator MS-2) visited Department of Molecular Biology and Biotechnology, Tezpur University, India (2019)
5. Prof. A.K. Mukherjee (Indian PI) visited Department of Pharmacology, University of Malaya, Malaysia (2019).

(Note: Due to Covid-19 pandemic, the exchange visits were restricted and final year visit could not be undertaken.)

B4. Details of Publications & Patents, if any:

B4.1. Publications in peer-reviewed international journal (Full funding from DST-ASEAN project which is mentioned in the acknowledgement section of the manuscript):

1. Patra, A., Banerjee, D., Dasgupta, S., & **Mukherjee, A. K.** (2021). The *in vitro* laboratory tests and mass spectrometry-assisted quality assessment of commercial polyvalent antivenom raised against the 'Big Four' venomous snakes of India. ***Toxicon***, *192*, 15-31.
2. Patra, A., & **Mukherjee, A. K.** (2021). Assessment of snakebite burdens, clinical features of envenomation, and strategies to improve snakebite management in Vietnam. ***Acta tropica***, 105833.
3. Patra, A., Herrera, M., Gutiérrez, J. M., & **Mukherjee, A. K.** (2021). The application of laboratory-based analytical tools and techniques for the quality assessment and improvement of commercial antivenoms used in the treatment of snakebite envenomation***. Drug Testing and Analysis*** doi: 10.1002/dta.3108. Epub ahead of print. PMID: 34089574. B.4.2.
4. **Mukherjee, A. K**., Mackessy, S. P. (2021) Prevention and improvement of clinical management of snakebite in Southern Asian countries: A proposed road map. ***Toxicon*** (revised manuscript communicated).

Publications in peer-reviewed international journal (partial funding from DST-ASEAN project which is mentioned in the acknowledgement section of the manuscript):

5. Patra, A., Kalita, B., Khadilkar, M. V., Salvi, N. C., Shelke, P. V., & **Mukherjee, A. K.** (2021). Assessment of quality and pre-clinical efficacy of a newly developed polyvalent antivenom against the medically important snakes of Sri Lanka. ***Scientific Report*** (accepted manuscript in press).
6. **Mukherjee, A. K.** (2020). Species-specific and geographical variation in venom composition of two major cobras in Indian subcontinent: Impact on polyvalent antivenom therapy. ***Toxicon***, *188*, 150-158.
7. Patra, A., & **Mukherjee, A. K.** (2020). Proteomic analysis of Sri Lanka *Echis carinatus* Venom: immunological cross-reactivity and enzyme neutralization potency of Indian polyantivenom. ***Journal of Proteome Research***, *19*(8), 3022-3032.
8. **Mukherjee, A. K**., Chanda, A., & Patra, A. (2020). Species-specific and geographical variation in venome compostion of Indian cobra: Impact on polyantivenom therapy. ***Toxicon***, *182*, S3-S4.
9. Chanda, A., & **Mukherjee, A. K.** (2020). Quantitative proteomics to reveal the composition of Southern India spectacled cobra (*Naja naja*) venom and its immunological cross-reactivity towards commercial antivenom. ***International Journal of Biological Macromolecules****, 160, 224-232*.
10. Patra, A., Chanda, A., & **Mukherjee, A. K.** (2019). Quantitative proteomic analysis of venom from Southern India common krait (*Bungarus caeruleus*) and identification of poorly immunogenic toxins by immune-profiling against commercial antivenom. ***Expert review of proteomics***, *16*(5), 457-469.

11. Chanda, A., Kalita, B., Patra, A., Senevirathne, W. D. S. T., & **Mukherjee, A. K.** (2019). Proteomic analysis and antivenomics study of Western India *Naja naja* venom: correlation between venom composition and clinical manifestations of cobra bite in this region. ***Expert Review of Proteomics***, *16*(2), 171-184.
12. Chanda, A., Patra, A., Kalita, B., & **Mukherjee, A. K.** (2018). Proteomics analysis to compare the venom composition between *Naja naja* and *Naja kaouthia* from the same geographical location of eastern India: Correlation with pathophysiology of envenomation and immunological cross-reactivity towards commercial polyantivenom. ***Expert Review of Proteomics***, 15 (11), 949-961.
13. Kalita, B., Patra, A., Das, A., & **Mukherjee, A. K.** (2018). Proteomic analysis and immuno-profiling of eastern India Russell's viper (*Daboia russelii*) venom: Correlation between RVV composition and clinical manifestations post RV bite. ***Journal of Proteome Research****,* 17 (8), 2819-2833.
14. Kalita, B., Singh, S., Patra, A., & **Mukherjee, A. K.** (2018). Quantitative proteomic analysis and antivenom study revealing that neurotoxic phospholipase A_2 enzymes, the major toxin class of Russell's viper venom from southern India, shows the least immuno-recognition and neutralization by commercial polyvalent antivenom. ***International Journal of Biological Macromolecules*** 118 (A), 375-385.

B4.2. Conference presentation:

1. **Mukherjee, A. K.,** Chanda, A., & Patra, A. (2020) 'Species-specific and geographical variation in venom composition of Indian cobra: Impact on polyantivenom therapy' on "**Venom week 2020**", organized by North American Society of Toxinology, at Gainesville, Florida, USA from 04- 07 March, 2020.
2. **Mukherjee, A. K.,** and Patra A., (2018) 'Assessment of quality of antivenom for efficient hospital management of snakebite' on the 2nd Circular National Conference on "**Recent Advances in Applied Biological Sciences**", NEHU, Shillong, India, from 04- 05 May, 2018.
3. **Mukherjee, A. K.,** Patra, A., Kalita, B., Chanda, A. (2019) 'Assessment of efficacy and quality control of commercial antivenom for efficient hospital management of snakebite patients' on "**National Toxicology Conference 2018**", Hanoi, Vietnam.

A significant achievement:

Prof. Ashis K. Mukherjee, the PI of this project is selected as an **Expert Member, World Health Organization (WHO),** committee under Neglected Tropical Diseases for control and prevention of snakebite envenoming.

Ashis Kr. Mukherjee

Dated: 09 July, 2021

(A. K. Mukherjee)
Principal Investigator

GFR 12 – A

[(See Rule 238 1))]
UTILIZATION CERTIFICATE (UC) FOR THE YEAR 2020-2021
in respect of *RECURRING*
as on 31st March 2021 to be submitted to SERB

Is the UC *(Provisional/~~Audited~~)*
(To be given separately for each financial year ending on 31st March)

1. Name of the grant receiving Organization : Tezpur University
2. Name of Principal Investigator(PI): Prof. Ashis K Mukherjee
3. SERB Sanction order no. & date: IMRC/AISTDF/R&D/P-3/2017, dated 1st February, 2018
4. Title of the Project: "Studies on epidemiology, hospital management of snakebite, and standardization of laboratory tests for assessment of efficacy and quality control of commercial antivenom manufactured in India and in ASEAN countries"
5. Name of the SERB Scheme: AISTDF, ASEAN-India collaborative research project (CRG/NPDF/ECR etc.)
6. Whether recurring or non-recurring grants: ***RECURRING***
7. Grants position at the beginning of the Financial year (Rupees)
   (i) Carry forward from previous financial year: INR 10,69,931.00
   (ii) Others, If any: NA
   (iii) Total: INR 10,69,931.00

8. Details of grants received, expenditure incurred and closing balances: (Actuals in Rupees)

Unspent balance of grants received previous years [figure as at Sl. No. 7(iii)]	Interest earned thereon 31st March 2021	Interest deposited back to the SERB	Grants received during the year April 2020 to March 2021		Total available funds (1+2-3+4)	Expenditure incurred (April 2020 to March 2021)	Closing Balance (5-6) as on 31st March 2020
1	2	3	4		5	6	7
			Sanction No (i)	Amount received (Rs.)			
10,69,931.00	9,667.00		IMRC/AISTDF/R&D/P-3/2017	5,30,000.00	16,09,598.00	4,65,622.00	11,43,976.00

9. Component wise utilization of grants: (Rupees)

Grants-in-aid- General	Grant-in-aid-creation for capital assets	Total
4,65,622.00	NA	4,65,622.00

10. Details of grants position at the end of the year (Rupees)

(i) Balance available at end of financial year: INR 11,43,976.00
(ii) Unspent balance refunded to SERB (If any): 11,43,976.00
(iii) Balance (Carried forward to next financial year) if applicable: NA

GFR 12 – A [(See Rule 238 (1))]
UTILIZATION CERTIFICATE (UC) FOR THE YEAR 2020-2021
in respect of *RECURRING*
as on 31st March 2021 to be submitted to SERB

Is the UC *(Provisional/~~Audited~~)*
(To be given separately for each financial year ending on 31st March)

Certified that I have satisfied that the conditions on which grants were sanctioned have been duly fulfilled/are being fulfilled and that I have exercised following checks to see that the money has been actually utilized for the purpose for which it was sanctioned:

(i) The main accounts and other subsidiary accounts and registers (including assets registers) are maintained as prescribed in the relevant Act/Rules/Standing instructions (mention the Act/Rules) and have been duly audited by designated auditors. The figures depicted above tally with the audited figures mentioned in financial statements/accounts.

(ii) There exist internal controls for safeguarding public funds/assets, watching outcomes and achievements of physical targets against the financial inputs, ensuring quality in asset creation etc. & the periodic evaluation of internal controls is exercised to ensure their effectiveness.

(iii) To the best of our knowledge and belief, no transactions have been entered that are in violation of relevant Act/Rules/standing instructions and scheme guidelines.

(iv) The responsibilities among the key functionaries for execution of the scheme have been assigned in clear terms and are not general in nature.

(v) The benefits were extended to the intended beneficiaries and only such areas/districts were covered where the scheme was intended to operate.

(vi) The expenditure on various components of the scheme was in the proportions authorized as per the scheme guidelines and terms and conditions of the grants-in-aid.

(vii) It has been ensured that the physical and financial performance under AISTDF, ASEAN-India collaborative research project (CRG/NPDF/ECR ..etc.) (Name of the scheme has been according to the requirements, as prescribed in the guidelines issued by Govt. of India and the performance/targets achieved statement for the year to which the utilization of the fund resulted in outcomes given at Annexure – I duly enclosed.

(viii) The utilization of the fund resulted in outcomes given at Annexure – II duly enclosed (to be formulated by the Ministry/Department concerned as per their requirements/specifications.)

(ix) Details of various schemes executed by the agency through grants-in-aid received from the same Ministry or from other Ministries is enclosed at Annexure –II (to be formulated by the Ministry/Department concerned as per their requirements/specifications).

Date.

Place: Tezpur

Signature of PI :	Signature with Seal :..... Name: ..... Chief Finance Officer (Head of Finance) Finance Officer Tezpur University	Signature with Seal..... Name: ..... Head of Organisation Registrar Tezpur University

(Strike out inapplicable terms)

REQUEST FOR ANNUAL INSTALMENT WITH UP-TO-DATE STATEMENT OF EXPENDITURE (1st April 2020 to 31st March 2021)

1. SERB Sanction Order No and date : IMRC/AISTDF/R&D/P-3/2017; dated 1st February 2018
2. Name of the PI : Prof. Ashis K. Mukherjee
3. Total Project Cost : INR 46,43,800.00
4. Revised project Cost (if applicable) : INR 48,27,550.00
5. Commencement: : 01/02/2018
6. Statement of expenditure : attached

(Month wise expenditure incurred during current financial year- April 2020 to March 2021)

Month & year	Expenditure incurred/ committed
April 2020	
May 2020	
June 2020	
July 2020	
August 2020	
September 2020	
October 2020	
November 2020	2,45,000.00
December 2020	85,114.00
January 2021	1,05,000.00
February 2021	14,883.00
March 2021	

7. Grant received in each year: (Rupees)

a. 1st Year : 15,47,000.00
b. 2nd Year : 10,33,098.00
c. 3rd Year : 5,30,000.00
d. Interest, if any : 21,572.00 + 21,572.00 +9,667 = 52,811.00

Total (a + b + c + d) : 31,62,909.00

Statement of Expenditure

(to be submitted financial year wise i.e. 1st April 2020 to 31st March 2021)

Sl. No (I)	Sanctioned Heads (II)	Total Funds Allocated (indicate sanctioned for 3rd year (1st April 2020 to 31st March 2021) (III)	Balance from the last financial year (2019-2020) (IV)	Total available funds during 1st April 2020 to 31st March 2021 [V = (III+IV)]	Expenditure incurred during 1st April 2020 to 31st March 2021 (VI)	Balance as on 31st March 2021 [VII = (V-VI)]	Interest earned during 1st April 2020 to 31st March 2021 (VIII)	Total balance (IX) IX =[VII+VIII]	Ramarks
									The balance amount will be refund to AISTDF Secretariat
1.	Manpower costs	3,50,000.00	-8400	3,41,600	3,50,000.00	-8,400.00			
2.	Research grant	1,00,000.00	45,146.00	1,45,146.00	85,114.00	45,149.00			
3.	Travel								
4.	Contingencies				14,883.00				
	International travel	65,787.00	9,54,213.00	10,20,000.00	0.00	10,20,000.00	9,667.00	11,43,976.00	
6.	Equipment	NA							
7.	Overhead expenses	13,175.00	13,175.00	26,350.00	15,625.00	10,725.00			
8.	Others (Not mentioned)	1,038.00	44,225.00	45,263.00	0.00	45,263.00			
9.	Interest earned as on 31st March 2020		21,572.00	21,572.00	0.00	21,572.00			
10.	Total	5,30,000.00	10,69,931.00	15,99,931.00	4,65,622.00	11,34,309.00	9,667.00	11,43,976.00	

Name and Signature of Principal Investigator:

Date:

Signature of Competent financial authority: _____
(with seal) Finance Officer Date: _____
Tezpur University

* DOS – Date of Start of project

Note:

1. Expenditure under the sanctioned heads at any point of time should not exceed funds allocated under that head without prior approval of SERB i.e