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Production of Polyvalent Region-Specific Antivenom

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Abstract: This study was carried out on horses belonging to the Holding Company for Biological Products and Vaccines (VACSERA). Two immunization protocols used for preparation of polyspecific horse antivenins were evaluated. The first and second immunization schedules followed by a rest period of about one month ended by re-immunization schedule. The antivenoms were raised to venoms of the four major snakes of South Sinai (three vipers"*Cerastes cerastes, Echis coloratus, Pseudocerastes fieldi*" and the elapid '*Walterinnesia aegyptia*"). Therapeutical titers were obtained by applying the two immunization schedules however, a faster response was obtained by using a mixture of venoms rather than individual venoms. Furthermore, although the preparation of polyspecific antisera to a group of venoms is less costly, the data showed that it is practical to prepare polyspecific antivenom to a group of vipers not for both vipers and elapids. Eventually, the most logical procedure for preparation of polyspecific antisera to a group of vipers and elapids is by mixing antisera raised for vipers with another raised for elapids. So this work achieved the aims of producing potent polyvalent antivenom suitable for use in a specific area and replacing the already present technique (*in vitro* mixing of monospecific antivenom prepared separately in horses) by immunization of horses with the different venoms as well as preparation of polyspecific antivenom from single horse.

Key words: Snakes · Antivenom · Polyvalent · Region-Specific

INTRODUCTION

Snakebites represent a serious public health problem in many areas of the world. Traditional treatment is sought by most victims. Many survivors of snakebites are left permanently maimed. The African savanna region, South Africa, the Indian subcontinent, South East Asia, China, New Guinea and Central / South America are the worst affected. Community-based studies have shown that between 4 and 162 annual snakebites fatalities per 100,000 population. Unlike most classical tropical diseases, snakebites are increasing, due to man made environmental changes, which encourage colonization of populated areas by new and more dangerous species. The clinical history of snakebite is usually unambiguous, but the snake responsible is rarely brought. In humans, pathophysiological mechanisms of envenoming include tissue damage and inflammation, cardiovascular disturbances and haemorrhagic diathese. Some venom toxins rapidly cause irreversible effects.

Around the world, there are many different antivenoms available, most of which are produced against single venoms and are only effective against those specific venoms (monovalent or monospecific antivenoms). There are also antivenoms made by immunizing the same animal using several venoms from different snake species or combining the serum antibodies from several animals immunized against the venom of particular snake species. These so-called "Poly-valent or polyspecific antivenoms" have a broader spectrum of activity [1].

The term "monospecific antivenom" refers to an antivenom raised against the venom of a single species or sub-species of a venomous animal. The term "polyspecific antivenom" refers to an antivenom raised against a mixture of two or more venoms from different species or sub-species of a venomous animal [2].

The critical problems associated with supply and distribution of antivenoms in Sub-Saharan Africa were reviewed by Theakston *et al*. [3]. In Africa, snakebites

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cause more than one thousand deaths each year and thousands of cases of permanent physical disability. *Echis* (saw-scaled viper), *Naja* (cobras) and *Bitis arietans* (puff adders) are the most important species. In the past, two large European companies produced polyspecific antivenoms suitable for Africa, supplementing the production of antivenoms from the South Africa Institute of Medical Research (SAIMR). Even so, an insufficient antivenom was distributed to treat more than a fraction of African snakebite victims. In recent years, the situation has become more acute since one of the European manufacturers has stopped production entirely, whilst the other has only very recently resumed limited production after revising its production method; the SAIMR has been privatised. Other antivenom manufacturers have raised concerns about costs. As a result, the burden of human suffering from snakebite in Africa, including mortality, is increasing. The choice is now between imported, ineffective, nonspecific antivenoms manufactured using geographically inappropriate venoms and unproven and frequently dangerous traditional treatments [3]. At the very least, antivenoms should be tested for neutralizing ability against the venoms of all major populations of any species, with special emphasis on those occurring in regions with a high incidence rate of snakebite.

The current study was carried out as a trial to produce potent polyvalent antivenom suitable for use in a specific area.

MATERIALS AND METHODS

Animals

Horses: Four adult male horses belonged to laboratory animal farm of Egyptian Organization for Biological Products and Vaccines (VACSERA) were used for the production of polyvalent antivenom in this study. Horses aged between 3-4 years, each weighting about 300-350 kg, Animals were daily fed on 3 kg barley, 2 kg wheat straw and ½ kg bran with hay or barseem. Animals had not been injected with snake venoms prior to this study. The used

Mice: Mice, each weighting 16-18 g, obtained from the Laboratory Animal Unit of Helwan Farm-VACSERA, were used for determination of LD_{50} of different venoms used and also in venom-neutralization assay for titration of the antivenom antibody level in horse sera.

Vipers and Snakes: Vipers and snakes belonging to South Sinai province [4] were used in this experiment. These include *Cerastes cerastes* (, *Pseudocerastes fieldi*,

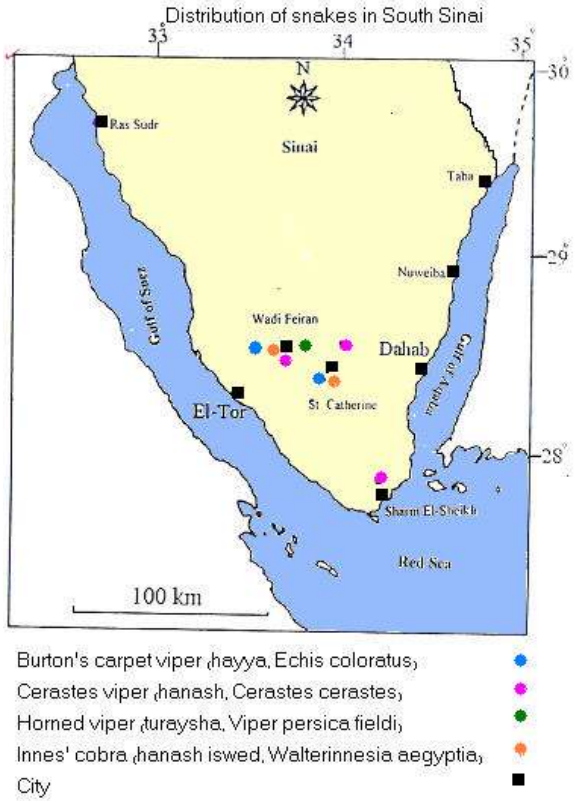

Photo 1: Distribution of snakes and vipers in South Sinai [4]

Echis coloratus and *Walterinnesia aegyptia*. They are distributed, as shown in Photo (1) in different areas of Sinai province. They were collected from the area of choice and kept in Helwan Serpentarium to extract their venoms in a process called "milking", which is simple but skilled operation [5].

Venoms: Venoms were collected from adult snakes and vipers isolated from the South Sinai province and kept at the Serpentarium of the Venoms Production Center in Helwan farm-VACSERA. The process of venom collection is called milking. This process is carried out by seizing the snake correctly behind the head and then opening its mouth or inducing it to bite through a membrane into a small cup. While the fangs are in the cup, the head of the snake is pressed gently to force venom from the glands down the fangs. The extracted venom was freeze-dried using freeze-drying apparatus to obtain venom in a powder form and stored at-20°C [6].

Determination of Lethal Dose$_{50}$ (LD_{50}) of Used Venoms: The lethal dose 50 (LD_{50}) was measured according to Spearman and Kärber [7,8] for *Cerastes cerastes,*

Table 1: The first immunization schedule in horses number 879 and 880

Dose Number	Weeks	*Cerastes cerases**	*Pseudocerastes fieldi**	*Echis coloratus**	*Walterinnesia aegyptia**	Adjuvant
Primary Immunization						
1	0	3	-	-	-	With CFA
2	2	-	3	-	-	With CFA
3	4	-	-	3	-	With CFA
4	6	-	-	-	3	With CFA
Secondary Immunization						
5	8	2	2	2	3	-
6	10	4	4	4	6	-
7	12	5	5	6	10	-

*Weights of venom by mg.

Table 2: The second immunization schedule in horses number 881 and 882

Dose Number	WeeKS	*Cerastes cerastes**	*Pseudocerastes fieldi**	*Echis coloratus**	*Walterinnesia aegyptia**	Adjuvant
Primary Immunization						
1	0	1	1	1	1	With CFA
Secondary Immunization						
2	2	2	2	2	2	With CFA
3	4	3	3	3	3	With CFA
4	6	3	3	3	5	With CFA
5	8	3	3	3	5	-
6	10	5	5	6	8	-
7	12	5	5	6	10	-

*Weights of venom by mg.

Pseudocerastes fieldi, Echis coloratus and *Walterinnesia aegyptia* using albino Swiss mice 16-18 g body weight.

Preparation of Venom for Injection: The used venoms were dissolved in 2 ml saline solution and used as they are or emulsified in Complete Freund's adjuvant according to the immunization protocol.

Immunization of Horses: Immunization of horses was carried out as outlined by MacCall [9].

Experiment Design: Two immunization protocols were used for immunization of horses against the four selected types of the venoms. The two immunization protocols or schedules were compared for determination of the best immunization protocol.

The First Immunization Schedule: In this protocol, 2 horses (number 879 and 880) were used and immunized according to the schedule shown in Table 1. The two horses were injected subcutaneously with one of the four types one time at 2 weeks intervals starting with *Cerastes cerastes* then *Pseudocerastes fieldi* followed by *Echis coloratus* and finally *Walterinnesia aegyptia*. Each venom dose contained 3 mg concentration emulsified with CFA.

Wo weeks after the injection of the fourth venom [day 55] secondary immunization with a mixture of the 4 venoms (polyvenom) without adjuvant was carried out. Three polyvenom doses were given at 2 weeks intervals. The first polyvenom dose contained 2 mg from each venom type. The second dose contained 4 mg from the first three and 6 mg of *Walterinnesia aegyptia*. The third dose consisted of 5 mg of *Cerastes cerastes* and *Pseudocerastes fieldi*, 6 mg of *Echis coloratus* and 10 mg of *Walterinnesia aegyptia.*

Using *in-vivo* venom neutralization test in mice, the sera potency designated as (the number of LD_{50} neutralized by 1.0 ml of the antivenom)was determined before and after immunization.

Table 3: The re-immunization schedule in horses

Dose Number	weeks	*Cerastes cerases**	*Pseudocerastes fieldi**	*Echis coloratus**	*Walterinnesia aegyptia**	Adjuvant
1	16	2	2	2	5	With CFA
2	17	2	5	5	10	With CFA

Weights of venom by mg.

The Second Immunization Schedule: This immunization protocol was applied for another two horses (number 881 and 882) as shown in Table 2. In this protocol, the immunized horses were subjected to a primary and secondary immunizations using polyvenom injection (mixture of the 4 venom types) from the first dose. The immunization was done by injection of the polyvenom subcutaneously at 2 weeks intervals. The first dose contained 1 mg from each of the 4 types, namely *Cerastes cerastes, Pseudocerastes fieldi, Echis coloratus* and *Walterinnesia aegyptia*, respectively, emulsified in CFA. The second dose composed of 2 mg from each type of the venom emulsified in CFA. The third dose contained 3 mg from each type, emulsified in CFA. The fourth dose contained 3 mg from the first three types and 5 mg from *Walterinnesia aegyptia* emulsified in CFA.

From the fifth dose upwards the polyvenom was injected without adjuvant. The fifth dose was similar in venom concentration to the 4^{th} dose. The sixth dose composed of 5 mg from both of *Cerastes cerastes, Pseudocerastes fieldi*, 6 mg of *Echis coloratus* and 8 mg of *Walterinnesia aegyptia*. The 7^{TH} dose was similar to the 6^{th} dose except *Walterinnesia aegyptia* was 10 mg.

The antibody titers were determined by *in vivo* venom-neutralization test in mice and were designated as (the number of LD_{50} neutralized by 1.0 ml of the antivenom).

Studies on the Effect of Re-immunization on the Antivenom Antibody Titers in the Two Immunization Protocols: The immunized horses in both protocols (Table 3) were injected subcutaneously with the polyvenom mixture emulsified in CFA. Two doses emulsified in CFA were given at one week interval as follows:

- The first dose composed of 2 mg from each of *Cerastes cerastes, Pseudocerastes fieldi* and *Echis coloratus* and 5 mg of *Walterinnesia aegyptia*.
- The second dose contained 2 mg of *Cerastes cerastes*, 5 mg of *Pseudocerastes fieldi* and *Echis coloratus* and 10 mg of *Walterinnesia aegyptia*.

The antivenom antibody titers were measured using the *in-vivo* venom neutralization assay at the end of the immunization period and the antibody titers were designated as the number of LD_{50} neutralized by 1.0 ml of the antivenom.

The *In-vivo* Venom-neutralization Test: It is used for determination of antibody titer against each type of the venoms used. The antivenom antibody titer was expressed as the number of venom LD_{50} neutralized by 1.0 cc of antivenom [1].

The Antibody Titration Level for the Produced Monospecific Antivenom in Helwan Farm Was as Follows:

- *Cerastes cerastes*: 80-130 LD_{50}/ 1 ml serum
- *Pseudocerastes fieldi*: 100-150 LD_{50}/ 1 ml serum
- *Echis coloratus*: 35-55 LD_{50}/ 1 ml serum
- *Walterinnesia aegyptia*: 35-45 LD_{50}/ 1 ml serum

Evaluation of the Health Status of the Envenomated Horses: Blood and serum samples were taken at the onset of immunization as well as before bleeding of re-immunization schedule.

Liver Function Test:

- Serum GOT activity was determined according to Reitman and Frankel [10].
- Serum total bilirubin value was determined according to Jendrassik and Grof [11].

Kidney Function Test:

- Serum creatinine value was determined according to Newman and Price [12].

Haemoglobin Determination: Haemoglobin was determined according to Van Kampen and Zijlstra [13].

Data were computed and statistically analizad [14].

RESULTS

Results of the Venoms Potency: Table 4 shows the lethality of venoms as determined by Spearman-kärber method for the four types of venom in mice.

Table 4: Results of venom potency in mice

Venom	LD_{50}µg/mice
Cerastes cerastes	9.2
Pseudocerastes fieldi	5
Echis coloratus	30
Walterinnesia aegyptia	9.2

Table 5: Results of antivenom antibodies titration* in horses (number 879 and 880) immunized using the first immunization schedule

		Cerastes cerastes		*Pseudocerastes fieldi*		*Echis coloratus*		*Walterinnesiaaegyptia*	
Serum sample number	Day	879	880	879	880	879	880	879	880
1	13	15	15	-	-	-	-	-	-
2	27	↓30	↑30	↓30	20	-	-	-	-
3	41	↑30	↑30	↓30	↓30	20	20	-	-
4	55	50	50	↓30	↑30	20	20	↓15	↓15
5	69	80	80	↓50	↓50	30	30	↓15	↓15
6	83	↑80	↑80	↑70	↑70	50	50	↓15	↓15
7	97	120	120	100	100	90	80	↓15	↓15

* Titration was done using *in vivo* neutralization assay

The antibody titer is expressed as antisera potency. It is the number of LD_{50} that can be neutralized by 1 ml of antiserum.

↓: less than, ↑: more than

Table 6: Results of antivenom antibodies titration* in horses (number 881 and 882) immunized using the second immunization schedule

		Cerastes cerastes		*Pseudocerastes fieldi*		*Echis coloratus*		*Walterinnesiaaegyptia*	
Serum sample number	Day	881	882	881	882	881	882	881	882
1	13	15	15	20	↓20	↓20	↓20	↓15	↓15
2	27	↑30	↑30	35	↓30	↓20	↓20	↓15	↓15
3	41	↑50	↑50	↓50	30	↑20	25	↓15	↓15
4	55	70	70	70	↓70	↓70	↓70	↓15	↓15
5	69	80	80	70	70	70	↑70	↓15	↓25
6	83	↑80	↑80	90	90	↑70	↑70	↓15	15
7	97	100	100	90	100	80	100	↓15	15

* The antibody titration was done using *in vivo* neutralization assay.

Unit of the sample titration result was LD_{50}.

The antibody titer is expressed as antisera potency. It is the number of LD_{50} that can be neutralized by 1 ml of antiserum.

↓: less than, ↑: more than

Results of the First Immunization Schedule: The produced antivenom antibodies measured by using *in vivo* neutralization assay and the results were presented in Table 5 and Fig. 1. Following the polyvalent immunization gradual increase in the antibody titers were recorded for each venom type except for *Walterinnesia aegyptia*. Two weeks after the last booster dose (day 97) the antibody titers reached 120, 100, 90 and 15 LD_{50}, respectively. Exceptionally *Walterinnesia aegyptia* showed no increase in the antibody titer during the whole immunization period inspite of the increase in the immunization dose.

Titration Results for First Immunization Schedule for Horses (Number 879 and 880):

The test used is *in-vivo* neutrlization assay

*the antibody titer is expressed as antisera potency. It is the number of ld_{50} that can be neutralized by 1 ml of antiserum.

Results of the Second Immunization Schedule: Results of antibody titers demonstrated in Table 6 and Fig. 2 showed gradual increase in the antibody titer for each type except for *Walterinnesia aegyptia* that manifested no increase. Two weeks after the fourth injection (day 55) the antibody titers reached 70, 70, 70 and 15 LD_{50} for *Cerastes cerastes, Pseudocerastes fieldi, Echis coloratus* and *Walterinnesia aegyptia,* respectively. Two weeks after the last dose (day 97) the antibody titers reached 100, 95, 90 and 15 $LD_{50,}$ respectively.

Titration Results for Second Immunization Schedule for Horses (Number 881 and 882)

Unit of the sample titration result was LD_{50}.

*The antibody titer is expressed as antisera potency. It is the number of LD_{50} that can be neutralized by 1 ml of antiserum

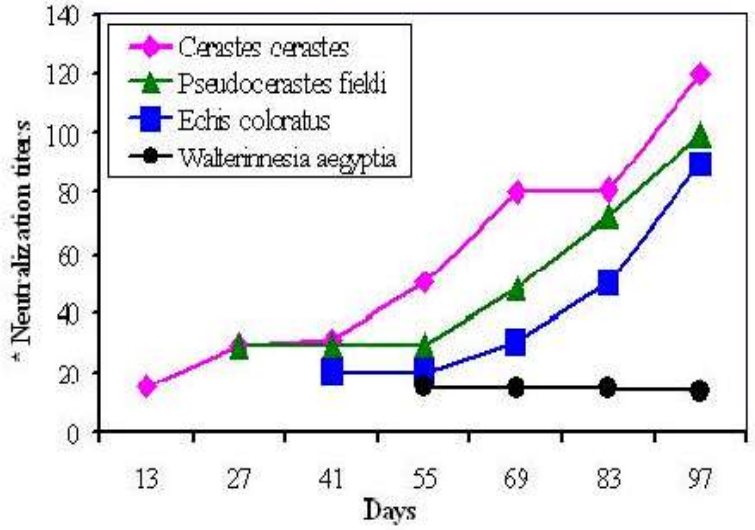

Fig. 1: Results of antivenom antibodies titration in horses (number 879 and 880) immunized using the first immunization schedule. Antibody titration was done using mice neutralization assay

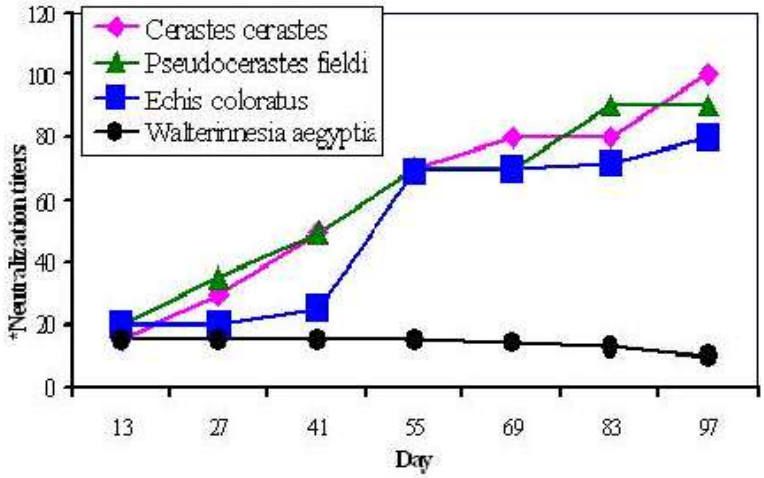

Fig. 2: Results of antivenom antibodies titration in horses (number 881 and 882) immunized using the second immunization schedule. Antibody titration was done using mice neutralization assay

Table 7: effect of re-immunization on the titer* of antivenom antibodies in horses (number 879 and 880)

		Cerastes cerastes		*Pseudocerastes fieldi*		*Echis coloratus*		*Walterinnesia aegyptia*		
Serum sample number		Day	879	880	879	880	879	880	879	880
1	0	↑80	70	70	60	60	↓50	↓15	↓15	
2	6	↑80	↑80	↑70	↓70	↓50	↓50	↓15	↓15	
3	13	100	90	70	70	50	↓50	↓15	↓15	

* Antibody titeration was done using *in vivo* neutralization assay
Unit of the sample titration result was LD_{50}.
The antibody titer is expressed as antisera potency. It is the number of LD_{50} that can be neutralized by 1 ml of antiserum.
↓: less than, ↑: more than

Table 8: Effect of re-immunization on the titer* of antivenom antibodies in horses (number 881 and 882)

Serum sample number	Day	*Cerastes cerastes*	*Pseudocerastes fieldi*	*Echis coloratus*	*Walterinnesia aegyptia*
0	0	↑80	↓70	↑50	↓15
1	6	80	↓70	↑50	↓15
2	13	120	90	70	↓15

* Antibody titeration was done using *in vivo* neutralization assay
Unit of the sample titration result was LD_{50}.
The antibody titer is expressed as antisera potency. It is the number of LD_{50} that can be neutralized by 1 ml of antiserrum

Table 9: Effect of envenomation on the health condition of the immunized horses

	Horse number			
	HB	Bilirubin	GOT	Creatinine
Normal value	11-19 g/dl	0.3-3.0 mg/dl	120-300 u/l	1.2-1.9 mg/dl
879				
Before injection	15.4	1.04	120	1.71
After re-immunization	16.5	1.6	130	1.1
880				
Before injection	14.8	1.42	160	1.94
After reimmunization	15.8	3.2	154	1.7
881				
Before injection	15.3	2.1	150	1.6
After reimmunization	15.3	1.3	143	1.3
882				
Before injection	13.2	1.78	125	1.54
After reimmunization	12.1	2.8	121	1.5

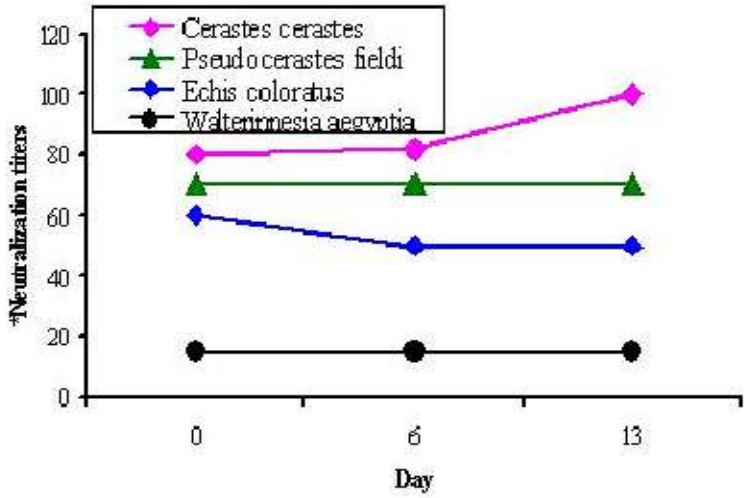

Fig. 3: Effect of re-immunization on the titer of antivenom antibodies in horses (number 879 and 880)

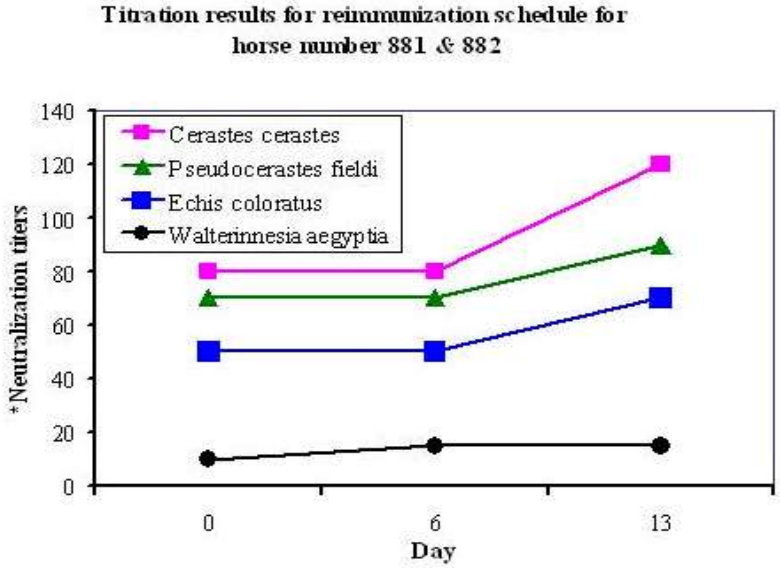

Fig. 4: Effect of re-immunization on the titer of antivenom antibodies in horses (number 881 and 882)

Effect of Re-immunization on the Titers Anti-venom Antibody in the Two Immunization Programs: In horses number 879 and 880, the antibody titers measured at the end of the re-immunization period were presented in Table 7 and Fig. 3. The obtained results manifested an increase in the antibody titers that reached 95, 70, 50 and 15 LD_{50} against *Cerastes cerastes, Pseudocerastes fieldi* and *Echis coloratus* and *Walterinnesia aegyptia*, respectively.

Titration Results for Re-immunization Schedule for Horses (Number 879 and 880)

Unit of the sample titration result was LD_{50}.

*The antibody titer is expressed as antisera potency. It is the number of LD_{50} that can be neutralized by 1 ml of antiserum.

In horses number 881 and 882, the re-immunization process manifested an increase in the antibody titers that reached at the end of the immunization to 120, 90, 70 and 15 LD_{50} against *Cerastes cerastes, Pseudocerastes fieldi* and *Echis coloratus* and *Walterinnesia aegyptia*, respectively (Table 8 and Fig. 4).

Titration Results for Re-immunization Schedule for Horses (Number 881 and 882)

Unit of the sample titration result was LD_{50}.

*The antibody titer is expressed as antisera potency. It is the number of LD_{50} that can be neutralized by 1 ml of antiserum

Effect of Envenomation on the Health Condition of the Immunized Horses: As shown in Table 9, the biochemical investigations included haemoglobin concentration, liver and kidney functions were within normal value for all horses before and after injections and bleedings.

DISCUSSION

Antivenom is crucial for systemic and severe local envenoming. Antivenom immunotherapy is the unique specific treatment of envenomation widely used and medically accepted. First produced by as monospecific antivenom, but due to unknown bites it was necessary to produce polyspecific type of antivenom covers all types in specific area.

The present work revealed the possibility of simultaneous immunization of horses with different types of snake venom for the production of polyspecific antivenom in horse with high titers of the respective specific antibodies. This suggests the existence of synergism between venoms used in the stimulation of the antibody response. Consequently, decreasing in the amount of venom used results in saving health condition of horse for more life production also decreasing in number of horses used and decrease protein concentration, which is obtained from mixing of different monospecific antivenom followed by concentration process to reduce the hypersensitivity problem in persons suffering from sensitivity against horse serum [15].

From the economic point of view, the study reduced the amount of venom used as in first immunization schedule the total venom injected were 65mg/horse divided as follows: 14 mg for both *Cerasates cerastes* and *Pseudocerastes fieldi*, 15 mg for *Echis coloratus* and 22 mg for *Walterinnesia aegyptia*. In the second immunization schedule total venom injected were 102 mg / horse divided as follows: 22 mg for both *Cerasates cerastes* and *Pseudocerastes fieldi*, 24 mg for *Echis coloratus* and 34 mg for *Walterinnesia aegyptia*. In the reimmunization schedule total venom injected were 33 mg / horse divided as follows: 4 mg for *Cerastes cerastes* and 7 mg for both *Pseudocerastes fieldi* and *Echis coloratus* and 15 mg for *Walterinnesia aegyptia* with number reduction of horse injected.

This work designed to inject 2 groups of horses with 4 types of venom. First group obtained the primary immunization through 4 separate injections, injected with monotype of venom at 2 weeks intervals with complete Freund's adjuvant followed by 3 injections as polyvenom with 2 weeks intervals as secondary immunization schedule. Second group obtained the primary immunization schedule as one shot of polyvenom followed by 6 doses of polyvenom as secondary immunization schedule at 2 weeks intervals with using complete Freund's Adjuvant in the first 4 doses only. After 2 weeks from finishing the injections, the results reached to the same therapeutical level of monospecific antivenom, so horses were bleed to obtain crude polyspecific plasma. Horses rested for about 1 month from the last injection followed by determination of antibodies level which remained after rest period.

These results clarify the use of low doses ranged from 1 to 10 mg for each venom and this agree with Soliman [16] who stated that very high and very low antigen doses are tolergenic, whereas the antigen-sensitive cells become non-reactive or even eliminated. Very high antigen doses are B-cell tolergenic and very low doses are T-cell tolergenic, however, in both cases the antibody production is impaired. The results also agree with Sriprapat *et al.* [17] who used the low dose,

low volume, multi-protocol. Between 1992 and 1995, an average of 2.597 g venoms was required to produce 100 L of antivenom. Between 1997 and 2001, the quantity was reduced to 0.276 g/100 L. or 10.6 % of what required previously.

Also using of adjuvant for 4 doses in the first and second immunization schedules and for 2 doses in the re-immunization schedule resulted in good immune response and this agrees with Chippaux and Goyffon [18] who mentioned that the use of immunological adjuvants is essential in antivenom production. Also, using of Complete Freund's Adjuvant (CFA) as an adjuvant of choice in the study achieved good immune response with notice of no granuloma formation, but the sterile abscess which was noticed appeared even in horses injected with venoms only out of the study. This disagrees with Christensen [19] who stated that Complete Freund's Adjuvant (CFA) is a very potent adjuvant but can cause serious side effects, i.e. sterile abscess and granuloma and its use in horses has been discouraged.

In the present study, using of multi-emulsion form CFA and injected subcutaneously with multi-site immunization, could help to be injected for many times and this agrees with Raw *et al.* [20] who tried to overcome this problem and minimize the local reactions through using of multi-emulsion form of CFA which used in Brazil. However, sterile abscess and granuloma could still be formed in about 25% of the horse, which also agrees with Pratanaphon *et al.* [21] who said that to avoid the serious local reactions caused by CFA at the injection sites a low dose, low volume, multi-site immunization protocol has been used with this simple immunization protocol, highly potent monovalent antivenom and polyvalent antivenom. This disagree with Christensen [22] who recommended the use of bentonite and aluminum phosphate in antivenom production to avoid the use of CFA. While, Sriprapat *et al*.[17]agreed to use CFA only once in each horse and clearly mentioned that an average of 14.5 immunization injections were needed when bentonite was used as compared to only 4.67 injections with the low dose, low volume, multi-site CFA protocol.

There are multiple routes for immunization. The route of choice along the experiment was subcutaneous route as it was suitable with adjuvant used and this agrees with Vaitukaitis [23] who cleared that CFA intradermally to horses could result in unacceptable local reactions at the injection sites. It also agree with Amyx [24]who selected the subcutaneous route as the most frequently used route due to the ease of injection technique. Larger volumes may be administered per site. Slower rate of absorption as the antigen is introduced primarily through the lymphatic system.However, using of monotype of venom and polyvenom doses in the study to produce polyspecific antivenom, the priming was 1 dose of mixed venoms or 4 doses in which each venom injected separetely resulted in good immune response. This agrees with Angulo *et al*. [6] who used a mixture of venoms for production of Crotalinae antivenom and disagrees with Theakston *et al*. [3] who mentioned that in contrast, the mixing of monospecific antivenom raised to different venoms in separate groups of animals. By producing these antisera separately, the number of possible antibody populations that is available for each antiserum is the same, but the number of epitopes in the immunogen is significantly less. Thus, it is postulated that the component antisera contains a higher proportion of protective antibodies against low molecular weight, poorly immunogenic components than polyspecific antivenoms. Combination of the monospecific antisera to produce a mixed monospecific antiserum results in an antivenom which has all the populations of the monospecific serum and therefore conveys better protection, but also has the advantages of a polyspecific antivenom in that the cross reactivity of the antivenom has been maximized. Although, a cross-protection is generally observed between closely related species, they are difficult to foretell and they necessitate individual verification. Furthermore, cross reactivity does not necessarily mean that there is cross-protection [25].

Good antibody response was achieved against 3 types of venom injected. This agrees with the observations of Gutierrez *et al*. [26] suggesting that when a horse gives a good antibody response against one of these venoms, it does the same against the other venoms included in the immunizing mixture.

Regarding the obtained results of biochemical parameters, immunization of horses with four types of venom which are *Cerastes cerastes, Pseudocerastes fieldi*, *Echis coloratus* and *Walterinnesia aegyptia* had resulted in no changes in chemical parameters of horses before and after injections. This may be due to low venom dosage used and healthy condition of newly introduced horses to the production line.

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