Journal of the Egyptian Society of Parasitology, Vol.52, No.2, August 2022
J. Egypt. Soc. Parasitol. (JESP), 52(2), 2022: 363 – 370
(Online: 2090-2549)

CROSS NEUTRALIZATION OF SOME KINDS OF VIPERS AND SNAKE VENOMS FROM AFRICA AND MIDDLE EAST USING VACSERA POLYVALENT VIPER ANTISERA

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Abstract

As the death rate due to snake bites was differ enormously between different countries, the study conducted was an extensive of neutralization of lethality of two species of genus *Naja*, seven species of genus *Vipera*, and two species of *Macrovipera* by using VACSERA equine antisera**.** The results showed that polyvalent snake venom antisera from VACSERA (which was prepared by injection of horses by *Cerrastes cerastes,* and *Echis carinatus*) was highly effective in neutralizing specifically to venom used for immunization and para-specifically to other species including *Naja haje, Naja nigricollis, Vipera palastinae, Vipera xanthina, Vipera ammodytes, Echis coloratus, Cerastes vipera and Pseudoechis* beside *Macrovipera* species including *Macrovipera lebetina obtuse, Macrovipera lebetina turanica*. The present study was established for whether specific or para-specific neutralization exists, its extent and the potency of para-specific versus specific neutralization within and between each genus, leading to wide spread of VACSERA Viper antivenom within the different countries.

Key words: Cross neutralization, Venoms, LD_{50}, ED_{50}, Elapidae, Vipredae, *Macrovipera*.

Introduction

As the death rate due to snake bites differed enormously, and how much information was accessible as a major factor, passage rates were from 0.13% to 4.8% in Egypt, Iran, Jordan, Morocco, Saudi Arabia, and Yemen (Dehghani *et al*, 2014; Vogel *et al*, 2018). Preparation of snake antivenom includes administration of the venom to a suitable animal mainly horses and after an appropriate period to collect the antibodies from their serum (Al-Shamsi *et al*, 2014). During such procedure the recipient animal may suffer different types of ill-health signs, such as generalized asthenia, pallor, skin rashes, muscular pain, hemorrhages, cardiovascular, respiratory problems, nervous signs as paresis and paralysis, break down of tissues, and finally collapse and death. The severity and duration of the clinical signs depend on the nature, amount and site of injected venoms (Minghui et *al,* 2019). Genus Vipera the widespread in Westernand Central Asia (Barbanera *et al,* 2009), is a genus in constantly recognized some two dozen species and a number of subspecies (Wüster, 1998*;* Garrigues *et al,* 2005; Joger *et al,* 2007; Thorpe *et al,* 2007; Wüster *et al,* 2008; Stümpel and Joger, 2009). The genus *Macrovipera* extends from Eastern Europe to Western and Central Asia, and in Mediterranean Africa (David and Ineich, 1999). From 1999 to 2008, several genus-level were established with transferred of some species of *Vipera* and *Macrovipera* to new genera *Daboia* or *Montivipera* (Reptile database, 2010).

In some instances, *Vipera* and *Macrovipera* venoms were the strongly inflammatory and necrotizing, as *Vipera* bites resulted neurotoxicity (Nashabaru *et al,* 2020). Para specificity (or cross-neutralization) refers to the capacity of an antivenom neutralized the venom of species, without including the immunization scheme of the animals used for anti-venom production at indicated therapeutically doses (Casasola *et al,* 2008). This was not excessively beyond the specific necessary for neutralization in some genera, and sometimes extends beyond a genus (Ramos-Cerrillo *et al,* 2008).

Para-specificity is determined in experimental animal, notably by neutralization of ve-

nom lethality, and extrapolating the results to clinical envenomation with careful cautions (WHO, 2010). However, systematic information of the bona fide spectrum of para-specific neutralization of lethality may be of use to treating clinicians in cases where the offending snake was not identified, or in cases where the offending species was identified but not included in the immunization protocol (Ursenbacher *et al,* 2008). The severity of envenomation, the resources available and other considerations, i.e. the expected safety of the antivenom and danger of sequelae even when symptomatic treatment would suffice to prevent death, must guide the choice to use antivenom in the absence of clinical validation of antivenom efficacy for particular species (Morais, 2018).

This study aimed to establish whether para-specific neutralization existed, its extent and the potency of para-specific versus specific neutralization within, and between each genus. Generated polyvalent experimental equine antisera used were the para-specific spectrum of protection afforded against a collection of seven *Vipera*, two *Macrovipera*, and two Elapidae venoms.

Material and Methods

Venoms*:* All venoms of *Naja haje, N. nigricollis, Vipera palastinae, V. xanthina, V. ammodytes, Echis coloratus, E. carinatus, Cerastes vipera* and *Pseudoechis,* also *Macrovipera lebetina obtuse* and *M. l. turanica* were prepared in lyophilized form at Helwan Farm, Egyvac as certified by ANDI, VACSERA. The venom was then dissolved in a sterile 0.9NaCl as1.0mg/ml.

Antivenom*:* Polyvalent viper venom antisera were prepared by horse's injection by special immunization schedule by *Cerastes cerastes and Echis carinatus* venoms and after an appropriate period collecting the specific antibodies from the plasma inoculated animal. VACSERA antivenom was a divalent antiserum raised by immunizing different groups of horses with special kind of venoms. Immunization scheme was the same for all groups, started with an initial dose of 2 mg/horse of each venom mixture emulsified with Complete and incomplete Freund's adjuvant (CFA, Rockland, PA) followed by doses venom without adjuvant. All immunizations were subcutaneous and antibody titers were monitored regularly (Elfiky *et al*, 2021). Experimental antiserum used was collected from horses by the plasmaphoresis technique, and consisted of equivolumetric pools of horse's plasma in each group

Animals: For lethal potency and neutralization of lethality, 20gm Albino Swiss mice (Vacsera) were used. All animal experimentation was carried out in accordance with the guide for the care and use of laboratory animals, which when with Helsinki's guidelines (WHO, 2010).

Lethal potency determination: Different doses of each venom species were injected IV in Albino Swiss mice (5mice/dose). The number of deaths 24hrs after injection was recorded; lethal potency was calculated as LD_{50} and dose of venom as µg/mouse that caused significant mortality was 50%. Pilot mortality versus venom dose was analyzed by using nonlinear regression (Casasola *et al,* 2008).

Lethality neutralization*:* Different doses of antivenom were incubated with five LD_{50} of each venom species for 30min at 37ºC. After incubation, samples were injected IV in mice (n ¼ 5/dose level). The number of deaths 48hr post-injection ED_{50} were calculated as the antivenom dose in microliters that protected 50% of mice. Anti-venom potency was calculated using the formula Potency ¼ [(n-1)/ED_{50}] LD_{50}, where n-1 represented number of lethal doses of challenge minus one. LD_{50} was subtracted from the total challenge dose (n) represented the dose that was theoretically responsible for the deathof half the mice, i.e. the calculation based on the total challenge minus one represents the actual quantity of venom that was otherwise responsible for 100%mortality and was thus neutralized by the antivenom as ED_{50} in ug/ul or (mg/ml) indicated the milligrams of venom neutralized by 1 ml of antivenom.

Statistical analysis: Data were presented as mean and standard deviation (±SD) or with 95% confidence intervals in parentheses. When indicated, Student's t-test was used for comparesons. Data were analyzed using the combined Prism 4.0 software package (GraphPad,CA, USA).

Results

Lethal potency of Cobra venoms: The most potent venom was that of Egyptian cobra*, N. haje* (2.1±0.2µg/mouce)*,* but spitting cobra, *N. nigricollis* v was (7.2±0.6µg/mouse).

Lethal potency of *Vipera* and *Macrovipera* venoms**:** All Vipera venoms were significantly more lethal than *Macrovipera* ones. The potent *V. ammodytes* was (8.25±0.9µg/ mouse) and the lowest one was *E. coloratus* (25±1.5µg/mouse). But, in *Macrovipera* venoms, *M. obtusa* was 18±1.2µg/mouse and *M. turanica* was 20.4±1.8µg/ mouse.

Neutralization of lethality of *cobra, Vipera* and *Macrovipera* venoms were neutralized, with specific potency of 200±10 ED_{50} *C. cerrastes*, 57±0.6 ED50 *E. carinatus*, but para-specific neutralization against cobra species was 75±1.5 ED_{50} *N. haje*, and 35±0.3 ED_{50} *N. nigricollis*, while para-specific neutralization against *Vipera* species was 15± 1.2 ED50 *V. ammodytes*, 40±0.4 ED_{50} *V. xanthine*, 65±1.5 ED_{50} *C. vipera*, 25±0.5 ED_{50} *V. palastinae*, 40±4.0 ED_{50} *E. coloratus*, 40±3.0 ED_{50} *Pseudocerastes feil- di*, 20±0.4 ED_{50} *M. l. obtuse*, 22±0.3 ED_{50} *M. l. turanica.*

Details were given in tables (1 & 2) and figures (1 & 2).

Table 1: Median lethal dose of venom of all venoms VACSERA Serpentarium

Venom	LD_{50}µg/20gm mouse	LD_{50} mg/kg (-)
Naja haje	2.1±0.2	0.105
Naja nigricollis	7.32±0.6	0.36
Cerastes cerastes	10.7±0.8	0.535
Vipera ammodytes ammodytes	8.25±0.9	0.412
Vipera xanthina	11.48±1.01	0.582
Cerrastes vipera	16±1.0	0.8
Vipera palastinae	19.1±1.1	0.95
Echis coloratus	25.5±1.5	1.25
Echis carinatus	20±0.8	1.02
Pseudo-cerastes feildi	21.25±1.8	1.06
Macrovipera lebatina obtuse	17.85±1.2	0.9
Macrovipera lebatina turanica	20.4±1.8	1.02

Table 2: Neutralization of lethality by polyvalent viper antivenom produced by vacsera, Egypt

Neutralization	Venom	*ED_{50} doses neutralized by 1ml polyvalent Viper antivenom
Specific	*Cerrastes cerrastes*	200±10.0
	Echis carinatus	57±0.6
Para-specific	*Naja Haje*	75±1.5
	Naja nigricollis	35±0.3
	Vipera ammodytes ammodytes	15 ±1.2
	Vipera xanthine	40±0.4
	Cerrastes vipera	65±1.5
	Vipera palastinae	25±0.5
	Echis coloratus	40±4.0
	Pseudo-cerastes feildi	40±3.0
	Macrovipera lebatina obtuse	20±0.4
	Macrovipera lebatina turanica	22±0.3

Discussion

Venom is an astounding transformative improvement that might be found all around the collection of animals. Human snakebites can have hazardous ramifications. As the current evaluations, venomous snakes are liable for up to 138, 000 deaths and up to 500, 000 instances of toxin-actuated sickness annually (Jenkins *et al,* 2021). Sero-therapy is right now the main successful treatment for envenoming. Polyclonal antibodies separated from the hyper immunized plasma are then bonded into the patient. An antiserum's avidity, specificity, and titer are three crucial qualities. The strength of an antiserum's antibodies' collaborations with an antigen is

estimated by its avidity. Titer of an antiserum was a definitive (ideal) measurement at which it is utilized in a strategy (Parveen *et al,* 2017). All sera depended on either IgGs or F(ab')2 pieces made by pepsin absorption of complete IgG antibodies to eliminate a large portion of the section crystallizable (Fc) district. F(ab')2 parts, similar to IgG antibodies are divalent because they have 2 antigen-restricting F (stomach muscle) areas consolidated by disulfide bonds (O´Leary and Isbister, 2009). Antivenom is an immunoglobulin [typically a pepsin-refined F (ab')2 fragment of full IgG] purified from the plasma of an immunized horse against the venoms of one or more snake or viper species (Archundia *et al,* 2011). Specific antivenom was developed specifically to neutralize snake venom bite, and neutralized venoms of closely related species or para-specific (Fathi *et al,* 2022).

Antivenom is monovalent that neutralized the venom of one snake type, but viper antivenom is polyvalent neutralized the venoms of multiple different snakes or viper species (Casewell *et al,* 2014). In order to assess the venom neutralizing efficacy of VACSERA antivenom, the venoms lethality was determined in mice. VACSERA polyvalent viper antisera were specifically neutralized by *C. cerastes*, and *E. carinarus* venoms. But, it was neutralized para-specifically by Elapidae, Vipidae and Macrovipera venoms.

In the present study, as to Elapidae venoms the LD_{50} of *N. haje* venom was 2.1µg/ mouse (0.105mg/kg) by IV injection. This nearly agreed by Seddik *et al.* (2002) and Shaban and Hafez (2003), they found that LD_{50} of *N. haje* venom was 0.2mg/kg by IP root, and 2.1µg/ mouse by IV root respectively. This difference may be due to different in route of injection. Also, the present LD_{50} of *Naja nigricollis* was 7.2µg/mouse (0.36mg/kg). This agreed with Abd El-Aziz *et al.* (2019), and Seddik *et al.* (2002), they found that LD_{50} of *N. nigricollis* was 0.34mg/kg and 5.5µg/mouse respectively in spite of the difference in injection root. But, it disagreed with Mosa *et al.* (2017) who found that it was 0.194mg/kg in rat. This difference may be due to difference in laboratory animals used.

In the present study, *Vipera* venoms were significantly more lethal than Macrovipera venoms as follows; LD_{50} of *C. cerrastes* venom was 10.7µg/mice (0.535mg/kg). This nearly agreed with Hassan and El-Hawary (1975) and Seddik et *al.* (2002) who found that it was 0.45mg/kg, and 9µg/mouse respectively. But, Mohamed *et al.* (1980) and Abd El-Aziz *et al.* (2019) found that it was 0.946 mg/kg, and 1.35mg/kg respectively by IP root. This difference may be due to the difference in route of injection. The present LD_{50} of *V. ammodytes* venom was 8.25µg/ mouse (0.412mg/kg). This agreed with both Archundia *et al.* (2011) and Garcia-Arredondo *et al.* (2019) who reported 8.4µg/ mouse, and 8.07µg/mouse respectively.

In the present study, LD_{50} of *V. xanthina* venom was 11.65µg/mouse (0.582mg/kg). This agreed with Archundia *et al.* (2011) who reported 12.2µg/mouse, and nearly agreed with Garcia-Arredondo *et al.* (2019) they found it was 7.03µg/mouse. Also, the present LD_{50} of *C. vipera* venom was 19.2µg/ mouse (0.9mg/kg). This nearly agreed with Seddik *et al.* (2002) and Saber *et al.* (2019) who found that it was 12.8µg/mouse, and 18.3µg/mouse (0.915mg/kg) respectively. Besides, the present LD_{50} of *V. palastinae* venom was 19µg/mouse (0.95 mg/ kg). But, it was 0.18mg/kg by Minton (1974), and 0.3 µg/gm (6.0µg/mouse) by Kochva, (1978), or 8.4µg/mouse by Archundia *et al.* (2011). These differences may be due to enviromental distributions.

In the present study, LD_{50} of *E. coloratus* venom was 25µg/mouse (1.25mg/kg). But, in the Sudan it was 20µg/mouse (Seddik *et al,* 2002). However, Casewell *et al.* (2010) reported that it was 9.81µg/mouse. This difference may be due to geographical distributions. Also, the present LD_{50} of *E. carinatus* was 20µg/mouse (1.25mg/kg). This more or less agreed with Abd El-Aziz *et al.* (2019)

who reported that it was 1.744 mg/kg, while it was 30µg/mouse in the Sudan species, and 25µg/mouse for the Saudi Arabian species (Seddik *et al,* 2002). Besides, the present LD_{50} of *P. fieldi* venom was 21.25µg/mouse (1.06mg/kg), but it was 6.0µg/mouse (Seddik *et al,* 2002).

In the present study, the LD_{50} of *Macrovipera lebatina* venom was 18µg/mouse (1.25mg/kg) for *Macrovipera obtusa*, and 20.4µg/mouse (1.02mg/kg) for *Macrovipera turanica*. These agreed with *Archundia et al.* (2011) who reported that it was 20.µg/mouse (1.02mg/kg) for *M. turanica*, and 30.1µg/mouse for *M. obtusa*. Also, the present result agreed with Warrell (1985) who reported that *Macrovipera l. obtuse* was 12-18µg /mouse, and Garcia-Arredondo *et al.* (2019) who found that it was 16.32µg/mouse for *M. obtusa*, and 18.36 µg/mouse for *M. turanica*. Nevertheless, Seddik *et al*. (2002) reported that it was 47µg/mouse without any specification.

In the present study, as to Elapidae venoms, 1ml of VACSERA Viper antisera neutralized para-specifically 75µl/mouse of venom *N. haje,* however, Ramos-Cerrillo *et al.* (2008) reported that it was 65.45 µl/mouse for Africa Elapidae antisera, and Harrison *et al.* (2017) reported 71.49µl/ mouse for SAIMR antisera. Also, 1 ml of VACSERA Viper antisera neutralized para-specifically35µl/mouse of venom *N. nigricollis,* and 71.49µl/mouse for SAIMR Elapidae antisera (Harrison *et al,* 2017). This difference may be due to differences in the antisera sources. Concerning Viperidae venoms, 1ml of VACSERA Viper antisera neutralized specifically *C. cerastes* by 200µl/mouse and *E. carinatus* by 57µl/mouse, and neutralized para-specifically *C. viper* by 65µl/mouse. But, it was neutralized para-specifically *V. ammodytes* by 15µl/mouse while it was 11.28µl/ mouse for Inoserp Europe antivenom (Garcia-Arredondo *et al,* 2019). VACSERA Viper antisera neutralized para-specifically *V. xanthina* by 40µl/mouse, but it was 16.13 µg/mouse for Inoserp Europe antivenom (Garcia-Arredondo *et al,* 2019).

In the present study, VACSERA Viper antisera neutralized para-specifically *V. palastinae* by 25µl/mouse, and it was 247 µg/mouse in specific *V. palastinae* antisera (Tirosh-Levy *et al,* 2019). Also it neutralized para-specifically *E. coloratus* by 40µl/ mouse, *P. cerastes feildi* by 40µl/mouse, *Macrovipera l. obtusa* by 20µl/mouse, and *M. l. turanica*by 22µl/mouse. These results reflected the antigenic difference between the specific venoms used in immunization and others not intended in immunization, or that venom elements responsible for lethality are antigenically conserved and wide spread via species/subspecies (Garrigues *et al,* 2005).

Conclusion

The results showed that VACSERA *Vip er* antivenom effectively neutralized the lethality of venoms specificity and para-specificity. *Vipera* venoms were significantly more lethal than *Macrovipera* ones. *Vipera* LD_{50} ranged from 8.25 to 25µg/ mouse, but *Macrovipera* ranged from 18-20.4µg/mouse. The Elapidae tested ranged from 2.1-7.2µg/mouse. But, VACSERA *Viper* antisera proved specifically and para-specifically neutrali-zed all *Vipera* and *Macrovipera* venoms tested ranged from 15 to 200 ED50/mouse, lowest para-specific neutralization potency was against *V. ammodytes* (15 ED_{50}/mouse). Tested Elapidae venoms ranged from 35 to 75 ED_{50} /mouse, but specific neutralizing ranged from 57.0 to 200 ED_{50} for *Echis carinatus* and *Cerrastes cerrastes*.

Consequently, the venom elements responsible for lethality were antigenically conserved and wide spread via species/ subspecies. This reflected the antigenic difference between the specific venoms used in immunization; in any case, the differences observed are in the limits of significance.

Recommendation

Try to wide spread of VACSERA *Viper*

antisera by evaluation of another species of snakes and vipers all over the worlds against it to determinate its efficacy.

Authors' contribution: All authors equally contributed in the field and laboratory work.

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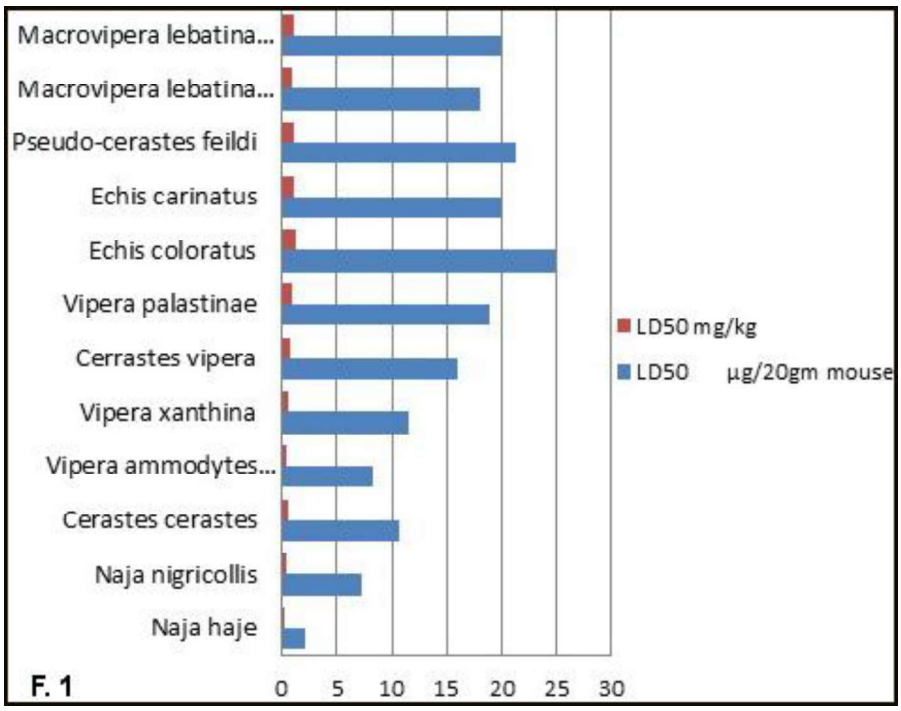
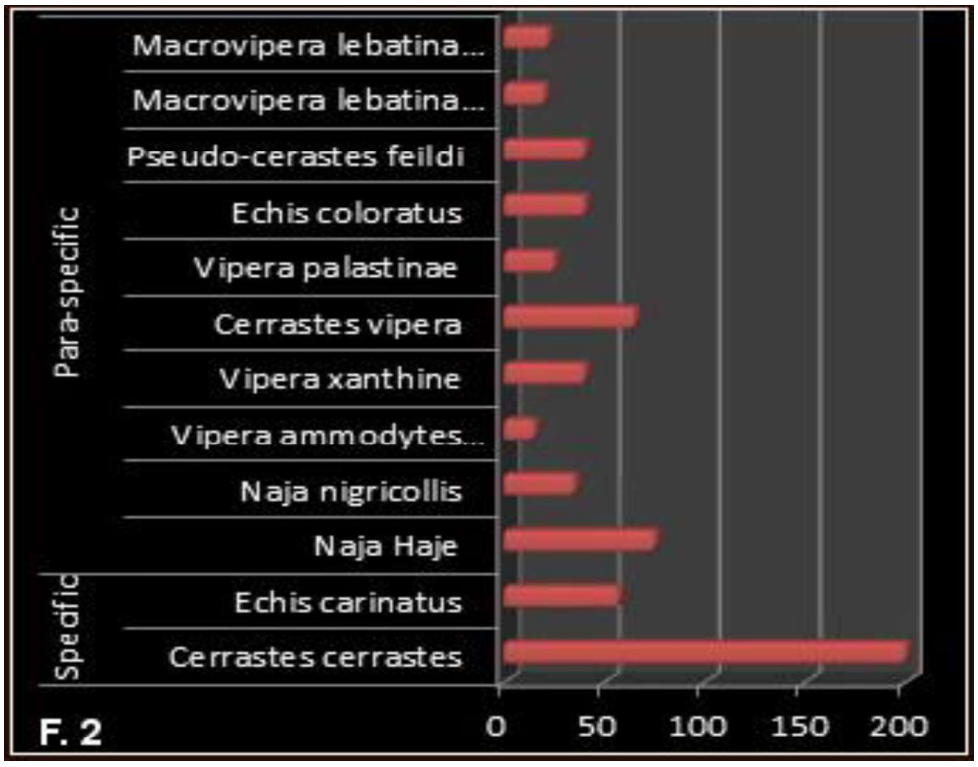
Explanation of figures

Fig. 1; Median lethal venom dose (LD_{50}) in all venoms.

Fig. 2: Neutralization of lethality by polyvalent snake anti-venom produced by VACSERA. *ED_{50} doses neutralized by 1ml polyvalent snake anti-venom.


Macrovipera lebatina...
Macrovipera lebatina...
Pseudo-cerastes feildi
Echis carinatus
Echis coloratus
Vipera palastinae
Cerrastes vipera
Vipera xanthina
Vipera ammodytes...
Cerastes cerastes
Naja nigricollis
Naja haje
LD50 mg/kg
LD50 µg/20gm mouse
0 5 10 15 20 25 30
F. 1


Para-specific
Specific
Macrovipera lebatina...
Macrovipera lebatina...
Pseudo-cerastes feildi
Echis coloratus
Vipera palastinae
Cerrastes vipera
Vipera xanthine
Vipera ammodytes...
Naja nigricollis
Naja Haje
Echis carinatus
Cerrastes cerrastes
0 50 100 150 200
F. 2