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Paraspecificity of *Vipera a. ammodytes*-specific antivenom towards *Montivipera raddei* and *Macrovipera lebetina obtusa* venoms

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ABSTRACT

Antivenom raised against the venom of nose-horned viper, *Vipera ammodytes* (*V. a.*) *ammodytes* (European viper venom antiserum, Zagreb antivenom), contains neutralising equine F(ab′)$_2$ fragments that are clinically successful against homologous venom, but also against the venoms of several others medically important European snakes due to its paraspecific action. In this work we demonstrated that Zagreb antivenom is preclinically effective in neutralising lethal toxicity and hemorrhagicity of venoms of Armenian mountain snakes – *Montivipera raddei* and *Macrovipera lebetina obtusa* as well. In order to better understand the biochemical basis of the observed paraspecificity, the ability of anti-*V. a. ammodytes* serum to recognise and neutralise proteinases of the two venoms was also investigated. Anti-*V. a. ammodytes* serum showed surprisingly low capacity to inhibit metalloproteinases of both venoms included in the study, probably due to weak immunorecognition of their P-I representatives. Also, it completely failed to abolish enzymatic action of serine proteinases from *Macrovipera lebetina obtusa* venom. Relevance of such finding is yet to be established.

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1. Introduction

Snake bite envenoming is a common and frequently devastating environmental and occupational disease, especially in rural areas of tropical developing countries, which is increasingly being recognised as a highly relevant public health issue on a global basis also (Chippaux, 1998a, 1998b; Theakston et al., 2003). Its specific treatment is critically dependent on the availability of safe and effective animal-derived antivenoms (Calvete et al., 2009). Production of antivenoms claims the adequate selection of snake specimens as candidates for collection of immunising venom mixtures which is generally based on recognising the ones that are responsible for the largest burden of envenoming in a particular geographic region, their range of distribution, and particularly, immunological relationships between their venoms and the venoms of other taxonomically related species (Gutiérrez et al., 2009). Namely, despite being subjected to accelerated molecular evolution, venoms of snakes belonging to the same or even different genera often tend to share antigenic determinants creditable for their immunoreactive cross-neutralisation, also known as paraspecificity (Calvete, 2010). Paraspecificity refers to the capacity of antivenom to neutralise the venom of the species not included in the immunisation mixture at therapeutically useful doses, *i.e.* not excessively beyond those necessary for specific neutralisation (Archundia et al., 2011). Animal experimentation, notably lethality neutralisation assay in mice as the golden

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standard for ensuring the antivenom potency, has usually been performed for paraspecific immunoreactivity determination (WHO, 2010). Additionally, more reliable assessment of the preclinical efficacy can be achieved by enrichment of such traditional analysis with other approaches measuring the ability of antivenom to abrogate the spectrum of toxic and enzymatic activities of probed venom, as well as by application of "antivenomics", a proteomics-based protocol for assessing the immunological profiles of antivenoms (Gutiérrez et al., 2009). Although extrapolation of the results obtained in laboratory to real clinical setting has to be undertaken with caution because of physiological limitations of *in vivo* and *in vitro* tests (Theakston et al., 2003; WHO, 2010), determination of paraspecific immunoreactivity may have important implications for antivenom design and use, especially in medical situations with urgent need for extension of neutralisation coverage to species whose venom is not included in the immunisation mixture (Calvete et al., 2009; Calvete, 2010). Additionally, knowledge concerning paraspecificity also may be of great significance for accessibility and supply improvements in regions experiencing inadequate coverage or complete lack of antivenoms produced against medically important venoms, an issue often associated with lack of epidemiological data, disorganisation of health services and high manufacturing costs in developing countries (Chippaux, 1998a).

Antivenom produced by hyperimmunisation of horses with the venom of nose-horned viper, *Vipera ammodytes* (*V. a.*) *ammodytes* (European viper venom antiserum, in the literature also known as Zagreb antivenom, Institute of Immunology Inc., Croatia), contains neutralising antibodies that are clinically successful against homologous venom, as well as against the venoms of several others medically important European snakes, as demonstrated by its continuous, over 30 years long use for the treatment of envenomings induced by *Vipera aspis* (Italy), *Vipera berus* (UK, Sweden), *Macrovipera lebetina* and *Montivipera xanthina* (Greece, Turkey). To possibly broaden its clinical coverage, we decided to screen paraspecificity exhibited by Zagreb antivenom towards the venoms of geographically more distant specimens by investigating the extent of their cross-neutralisation and chose *Montivipera* (*Mo.*) *raddei* and *Macrovipera lebetina* (*Ma. l.*) *obtusa*, both from the Armenian region, as a suitable candidates. These snakes are important cause of snake bite in western Asia each year, causing mild to severe local effects, such as tissue blistering, oedema, hemorrhage and necrosis, as well as hypotension shock and coagulopathy, especially clotting disorders and hypofibrinogenemia (Göçmen et al., 2006; Sanz et al., 2008; Ayvazyan and Ghazaryan, 2012). Although the venoms of *Mo. raddei* and *Ma. l. obtusa* have been thoroughly characterised at proteomic level (Sanz et al., 2008), their toxinological profile is less well studied and the information about the correlation with the clinical picture of envenoming still lacks. Despite high medical importance of those two Armenian mountain vipers it is noteworthy to mention that at the moment specific therapy against *Mo. raddei* envenoming, representing rare but potentially serious hazard, is not available. On the other hand, Zagreb antivenom shows sufficient level of protection against the venom of *Ma. lebetina*, as proved by its clinical effectiveness in treatment of snake bites occurring in Turkey. However, concerning the well documented phenomenon of geographical intraspecies variability of snake venoms in general, it would be of great importance to confirm therapeutic potential of Zagreb antivenom against bites of *Ma. lebetina* specimens from neighbouring regions as well.

To summarise, our objectives were i) to gain the information about the relevant biological and toxinological activities of *Mo. raddei* and *Ma. l. obtusa* venoms of Armenian origin; ii) to determine the degree of logical cross-reactivity between *V. a. ammodytes* and *Mo. raddei* venoms that has never been studied before and iii) to revise the preclinical efficacy of Zagreb antivenom against the venom of *Ma. l. obtusa*.

2. Materials and methods

2.1. Reagents and chemicals

Azocasein, Tween 20, ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Pefabloc) and N_α-benzoyl-L-phenylalanine-L-valine-L-arginine-*p*-nitroanilide hydrochloride, iodoacetamide and DL-dithiothreitol (DTT) were from Sigma–Aldrich, USA. Horseradish peroxidase-conjugated goat anti-rabbit IgG (HRP-anti-rabbit IgG) was from Bio-Rad Laboratories, USA. Chemicals for buffers and solutions were from Kemika, Croatia.

2.2. Snake venoms and antivenoms

Crude venom of *V. a. ammodytes* was collected by milking snakes kept at the Institute of Immunology, air dried at ambient temperature and stored in the dark at 4 °C until use. Crude *Ma. l. obtusa* and *Mo. raddei* venoms were supplied by Orbeli Institute of Physiology, National Academy of Sciences (Yerevan, Armenia) and stored at 4 °C until use. Commercial European viper venom antiserum (Zagreb antivenom) was from Institute of Immunology Inc., Croatia. Experimental rabbit serum against *V. a. ammodytes* venom (anti-venom serum) was produced according to production immunisation scheme on a small scale.

2.3. Animals for *in vivo* assays

All animal work was in accordance to the Croatian Law on Animal Welfare (2013) which strictly complies with EU Directive 2010/63/EU. Mice (18–20 g) used for the assays of lethal toxicity and neutralisation of lethal toxicity were of NIH Ola/Hsd strain, bred at the Institute of Immunology. Adult rats for the assays of hemorrhagic activity and neutralisation of hemorrhagic activity were of Lewis strain, also bred at the Institute of Immunology.

2.4. Assay of lethal toxicity

The lethal toxicity, expressed as the median lethal dose (LD_{50}), was determined according to method of Theakston

and Reid (1983) and European Pharmacopoeia (Ph.Eur.01/2008:0145). Experimental details are given in Lang Balija et al. (2005). Results are given as mean from at least five determinations ± standard error (SE).

2.5. Assay of neutralisation of lethal toxicity

The potential of four different production series of Zagreb antivenom to neutralise the lethal toxicity was determined in mice according to Ph.Eur.01/2008:0145, with modifications, as follows. Two-fold serial dilutions of Zagreb antivenom (1.2 mL), prepared in saline, were preincubated with equal volumes of the venom solution (20 LD_{50}/mL) for half an hour at 37 °C. The immunoprecipitates were removed by centrifugation at 3000 × g for 5 min. Clear supernatants were i.v. administered to group of four mice, each receiving 0.5 mL with 5 LD_{50} doses of venom. Deaths were recorded 24 h later. The median effective dose (ED_{50}), representing the amount of undiluted antiserum (in μL) capable of neutralising the lethality of applied venom dose in 50% of animals, was calculated by Spearman–Kärber method. Protective efficacy (R) was expressed as the number of LD_{50} doses that can be neutralised with 1 mL of undiluted Zagreb antivenom and was calculated by the equation $R = (Tv\text{-}1)/ED_{50}$. Tv represents the actual number of LD_{50} doses inoculated per mouse. Assay was performed at least two times and the results are expressed as mean ± SE.

2.6. Assay of hemorrhagic activity

Hemorrhagic activity, expressed as the minimal hemorrhagic dose (MHD), was evaluated according to Theakston and Reid (1983), with experimental details described by Lang Balija et al. (2005). Briefly, each rat on the dorsal side received 100 μL of saline containing venom prepared in doses ranging from 1.28 to 50 μg. After 24 h their skin was removed and from its inner surface the perpendicular diameters of the hemorrhagic lesions were measured. The minimum hemorrhagic dose (MHD) is defined as the least amount of venom (μg dry mass) which, when injected i.d., results in the lesion of 10 mm diameter. Every dose was tested on two animals. Results are given as mean from two independent determinations ± SE.

2.7. Assay of hemorrhagic activity neutralisation

Zagreb antivenom (diluted 2-, 5- or 10-fold) was incubated with equal volume of the venom solution (1 mg mL^{-1}) at 37 °C for 30 min. Aliquots of 100 μL were i.d. administered to group of two rats. As a negative control, saline instead of antivenom was used. The hemorrhagic lesions on the inner surface of the removed skin were observed 24 h later, their perpendicular diameters measured and corresponding surfaces calculated from which the average value for each dose was obtained. Results are expressed as $[(P_V - P_{V+AV})/P_V] \times 100 \pm SE$, where P_V represents the mean value of lesion surfaces induced by the venom alone and P_{V+AS} the mean value of lesion surfaces induced by the venom mixed with antivenom.

The potential of two-fold serial dilutions of Zagreb antivenom to neutralise the hemorrhagic activity induced by

2 MHD of each of the venoms included in the study was determined likewise.

2.8. Assays of proteolytic activity and proteolytic activity neutralisation

2.8.1. Assays of azocaseinolytic activity and azocaseinolytic activity neutralisation

Proteolytic activity was measured colorimetrically on azocasein as a substrate using the modified protocol of Jorge da Silva and Aird (2001), with modifications, as described in Kurtović et al. (2012). Additionally, proteolytic activity was also examined in the presence of inhibitors by preincubating crude venom with Na$_2$EDTA (5 mmol dm^{-3}) and/or Pefabloc (1 mmol dm^{-3} for Mo. raddei and V. a. ammodytes venoms and 2 mmol dm^{-3} for Ma. l. obtusa venom) at 37 °C half an hour prior to substrate addition. The velocity of the reaction obtained for intact venom was considered as 100% of the proteolytic activity. Results obtained for Na$_2$EDTA- and Pefabloc-inhibited venom were expressed in relation to it. All tests were performed two times and results are given as mean ± SE.

Azocaseinolytic activity neutralisation was assayed with serum obtained by immunisation of the rabbit with whole V. a. ammodytes venom, as follows. Two-fold diluted antivenom serum was preincubated with equal volume of intact venom solution (2 mg mL^{-1}) or venom solution treated with Pefabloc (2 mmol dm^{-3}). After removal of immunocomplexes, the residual proteolytic activity was estimated in supernatant aliquots (35 μL) as described above. Serum of the non-immunised rabbit served as a negative control. Results are expressed as mean from three independent determinations ± SE.

2.8.2. Assays of amidolytic activity and amidolytic activity neutralisation

Amidolytic activity was examined on N_α-benzoyl-L-Phe-L-Val-L-Arg-p-nitroanilide. The reaction mixture consisting of venom (0.1 mg mL^{-1} for Mo. raddei venom and 0.02 mg mL^{-1} for Ma. l. obtusa and V. a. ammodytes venoms) and chromogenic peptide substrate (0.2 mmol dm^{-3}) was prepared in 100 μL of 50 mM Tris/HCl buffer, pH 8.5. During incubation for up to 5 min at 37 °C, the absorbance was being measured at 382 nm ($A_{382\ nm}$) in 15 s intervals and plotted against the incubation time resulting in the linear relationship. The slope of the line represents the velocity of the enzymatic reaction and was used as a measure of amidolytic activity. Additionally, proteolytic activity was also examined in the presence of inhibitors by preincubating crude venom with Na$_2$EDTA (5 mmol dm^{-3}) and/or Pefabloc (1 mmol dm^{-3} for Mo. raddei and V. a. ammodytes venoms and 2 mmol dm^{-3} for Ma. l. obtusa venom) at 37 °C half an hour prior to substrate addition. The velocity of the reaction obtained for intact venom was considered as 100% of the proteolytic activity. Results obtained for Na$_2$EDTA- and Pefabloc-inhibited venom were expressed in relation to it. All tests were performed two times and results are given as mean ± SE.

Neutralisation of the amidolytic activity was performed by preincubating five-fold diluted anti-venom serum with equal volume of venom solution (0.2 mg mL^{-1} for Mo.

raddei venom and 0.04 mg mL^{-1} for *Ma. l. obtusa* and *V. a. ammodytes* venoms). After removal of immunocomplexes, the residual amidolytic activity was estimated in supernatant aliquots (50 μL) mixed with equal volume of N_{α}-benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide (0.2 mmol dm^{-3}). Serum of the non-immunised rabbit served as a negative control. Results are expressed as mean from three independent determinations ± SE.

2.9. 2D immunoblotting of venom proteins by Zagreb antivenom

Isoelectric focusing of venoms was performed in ZOOM IPGRunner Mini-Cell (Invitrogen, USA) using immobilised pH gradient (IPG) strips (7 cm long, non-linear pH 3–10) (Invitrogen, USA) previously rehydrated with protein sample (250 μg), according to the manufacturer's procedure. The following step voltage protocol was applied: 200 V for 20 min, 450 V for 15 min, 750 V for 15 min and 2000 V for 180 min. The focused IPG strips were equilibrated first with DTT (20 mmol dm^{-3}) and than with iodoacetamide (125 mmol dm^{-3}). Each equilibration step was performed at room temperature for 15 min. The second dimension of electrophoretic separation utilised SDS-PAGE analysis of proteins focused on IPG strip that was reduced and alkylated prior its loading to 4–12% Bis–Tris precast gel with MES as running buffer under reducing conditions in an Xcell Sure-Lock Mini-Cell (Invitrogen, USA). One set of gels was stained with acidic Coomassie Brilliant Blue R250 solution, while the other one was electro-blotted to the PVDF membrane, following the provided protocol (Invitrogen, USA). The blocking was performed with 5% (w/v) non-fat milk in PBS/T (0.05% (v/v) Tween 20 in PBS) buffer at 4 °C overnight. The blotted membrane was first incubated with Zagreb antivenom (1 000 000-fold diluted), than with in-house rabbit anti-horse F(ab′)$_2$ serum (10 000-fold diluted) and finally with HRP-anti-rabbit IgG (10 000-fold diluted). Each incubation step was performed at room temperature for 2 h. In between the membranes were thoroughly rinsed in PBS/T buffer. ECL Prime Western Blotting Detection Reagent was used for detection, according to the manufacturer's instructions (GE Healthcare, USA).

3. Results

3.1. In vivo neutralisation assays

The venoms of *Mo. raddei* and *Ma. l. obtusa* exerted lethal and hemorrhagic activities. Their LD$_{50}$ and MHD values, measured in mice and rats, respectively, are summarised in Table 1. *Mo. raddei* venom exhibited lethal toxicity comparable to that of *V. a. ammodytes* and showed the most pronounced hemorrhagic potential. *Ma. l. obtusa* venom proved as the weakest lethality- and hemorrhagicity-inducing agent.

As tested in mice, different production series of Zagreb antivenom had the highest protective efficacy against homologous venom, at the same time paraspecifically neutralising lower, but from regulatory point of view, satisfactory number of LD$_{50}$ doses of *Ma. l. obtusa* venom (Table 2). Comparably strong neutralisation activity was achieved in abolishing the lethality of *Mo. raddei* venom.

Table 1
The lethal toxicity (as LD$_{50}$ in μg) and hemorrhagic potency (as MHD in μg) of *V. a. ammodytes*, *Mo. raddei* and *Ma. l. obtusa* venoms. Results are given as mean ± SE.

	V. a. ammodytes	*Mo. raddei*	*Ma. l. obtusa*
LD$_{50}$ [μg]	4.4–13.7[a]	9.1 ± 0.5	18.4 ± 1.4
MHD [μg]	21.6–42.8[a]	6.0 ± 0.4	29.7 ± 0.4

[a] Depending on the geographical location (as determined by Halassy et al. (2011)).

The potential of Zagreb antivenom to neutralise hemorrhagicity of *Mo. raddei* and *Ma. l. obtusa* venoms tested in a dose of 50 μg was evaluated in rats. As expected, Zagreb antivenom exhibited the most effective protective power against hemorrhagic activity of *V. a. ammodytes* venom. It also undoubtedly showed neutralisation ability, although in a lesser degree, towards *Mo. raddei* and *Ma. l. obtusa* venoms (Fig. 1A). Namely, complete neutralisation of 50 μg of *V. a. ammodytes* venom was obtained with 2.5 μL of antivenom. Achievement of the same effect against the equal dose of *Mo. raddei* and *Ma. l. obtusa* venoms required its application in a volume of 25 μL. Therefore, Zagreb antivenom is approximately 10 times weaker in protecting from 50 μg of heterologes venoms in comparison to its neutralising potential against the same quantity of *V. a. ammodytes* venom.

Regarding significantly different hemorrhagicity of the venoms included in the study, the challenge dose of 50 μg corresponds to around 2.2 MHD of *V. a. ammmodytes*, 9 MHD of *Mo. raddei* and 1.6 MHD of *Ma. l. obtusa* venom. From this reason *in vivo* neutralisation assay was also performed with equal amounts of venoms in terms of their hemorrhagic activity, each of which contained approximately 2 MHD (or 12 μg of *Mo. raddei*, 60 μg of *Ma. l. obtusa* and 50 μg of *V. a. ammodytes* venom). Zagreb antivenom completely abrogated the induction of hemorrhagic lesions with *V. a. ammodytes* venom at 16-fold dilution (Fig. 1B). Complete neutralisation of *Mo. raddei* venom occurred at 8-fold and that of *Ma. l. obtusa* venom at 4-fold dilution.

In addition, it is interesting to mention that when testing neutralisation of hemorrhagicity elicited by higher doses (50 μg or approximately 9 MHD) of *Mo. raddei* venom, occurrence of two rings forming skin lesions was observed: smaller, inner ring with sharp edges and bigger, outer one with more diffused edges (Fig. 1C). According to the results, it seems that Zagreb antivenom ensures higher level of protection against hemorrhagins that produce inner ring, at the same time more weakly recognising the proteins responsible for the outer one. Those are probably present in very small quantities so their effect could not be observed when low doses of *Mo. raddei* venom were applied.

3.2. In vitro neutralisation assays

The venoms of *Mo. raddei* and *Ma. l. obtusa* were proteolytically active on azocasein (Fig. 2A). Similarly as with *V. a. ammodytes* venom, under experimental conditions used, Pefabloc (inhibitor of serine proteinases) inhibited approximately 40% and Na$_2$EDTA (inhibitor of Zn^{2+}-dependent metalloproteinases) around 60% of *Ma. l. obtusa* venom's proteolytic activity, while simultaneous

Table 2

The protective efficacies (R value or the number of LD_{50} doses that can be neutralised with 1 mL of undiluted serum) against the venom's lethal toxicity of different production series of Zagreb antivenom. Results are given as mean ± SE from at least two independent experiments. For *V. a. ammodytes* and *Ma. lebetina* venoms (from Latoxan) are given values obtained in routine quality control testing.

	R values for different Zagreb antivenom production series			
Mo. raddei	189.0 ± 8.0	202.0 ± 7.0	72.2 ± 17.0	123.5 ± 5.0
Ma. l. obtusa	128	n.d.	135.0 ± 21.5	47.8 ± 2.0
V. a. ammodytes	344	262	273	371
Ma. lebetina	56	92	107	53

administration of both inhibitors resulted in practically complete inhibition of azocaseinolysis. When the venom of *Mo. raddei* was used as a proteolytic agent, Pefabloc did not show any inhibiting effect. On the other hand, Na$_2$EDTA, either alone, either in combination with Pefabloc, completely abolished its enzymatic action on azocasein, suggesting that metalloproteinases solely are responsible for azocaseinolytic activity of *Mo. raddei* venom.

Proteolytic activity of *Mo. raddei* and *Ma. l. obtusa* venoms was also detected on N_α-benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide, representing a specific substrate for serine proteinases (Fig. 2B). Accordingly, Pefabloc proved as a highly efficient inhibitor of amidolysis exhibited by both tested venoms, while Na$_2$EDTA by itself completely failed to quench their proteolytic activity.

First set of *in vitro* neutralisation experiments was performed with intact venoms, using azocasein as a substrate. For practical reasons (strong opalescence of *m*-cresol from antivenom preparation, lack of suitable negative control *etc.*) their immunoreactivity was studied with anti-venom serum as Zagreb antivenom equivalent. Expectedly, four-fold diluted anti-venom serum was the most effective
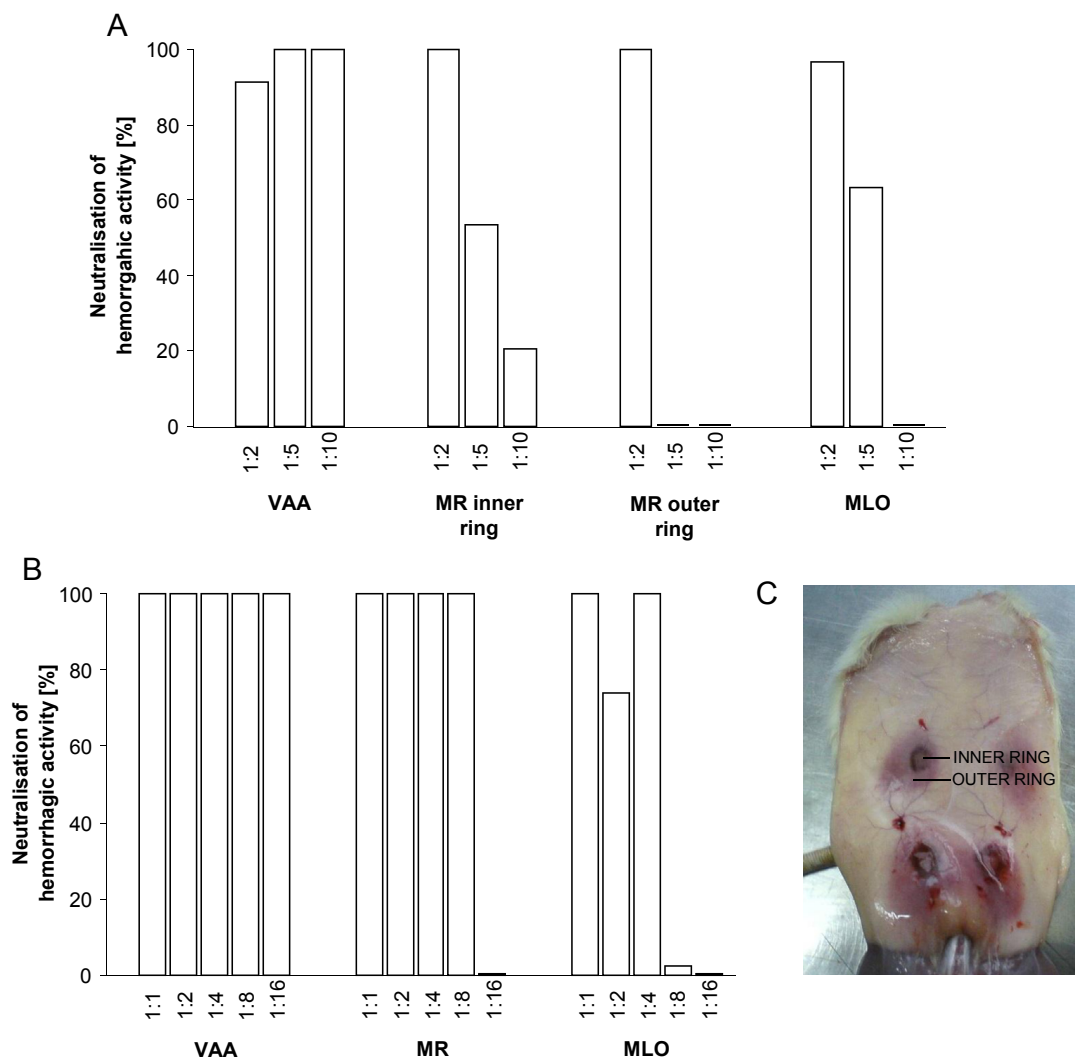

Fig. 1. The hemorrhagic activity neutralisation assay in rats performed with Zagreb antivenom. (A) Antivenom (diluted 1: 2, 1:5 or 1:10) was preincubated with 50 μg of *Mo. raddei* (MR), *Ma. l. obtusa* (MLO) or *V. a. ammodytes* (VAA) venom. (B) Antivenom (diluted 1: 1, 1:2, 1:4, 1:8 or 1:16) was preincubated with 2 MHD of *Mo. raddei* (MR), *Ma. l. obtusa* (MLO) or *V. a. ammodytes* (VAA) venom. (C) Representative result of neutralisation of hemorrhagicity elicited by 50 μg (or 9 MHD) of *Mo. raddei* venom where occurrence of smaller, inner ring with sharp edges and bigger, outer one can be observed. Zagreb antivenom ensures higher level of protection against hemorrhagins that produce inner ring, at the same time more weakly recognising the proteins responsible for the outer one.
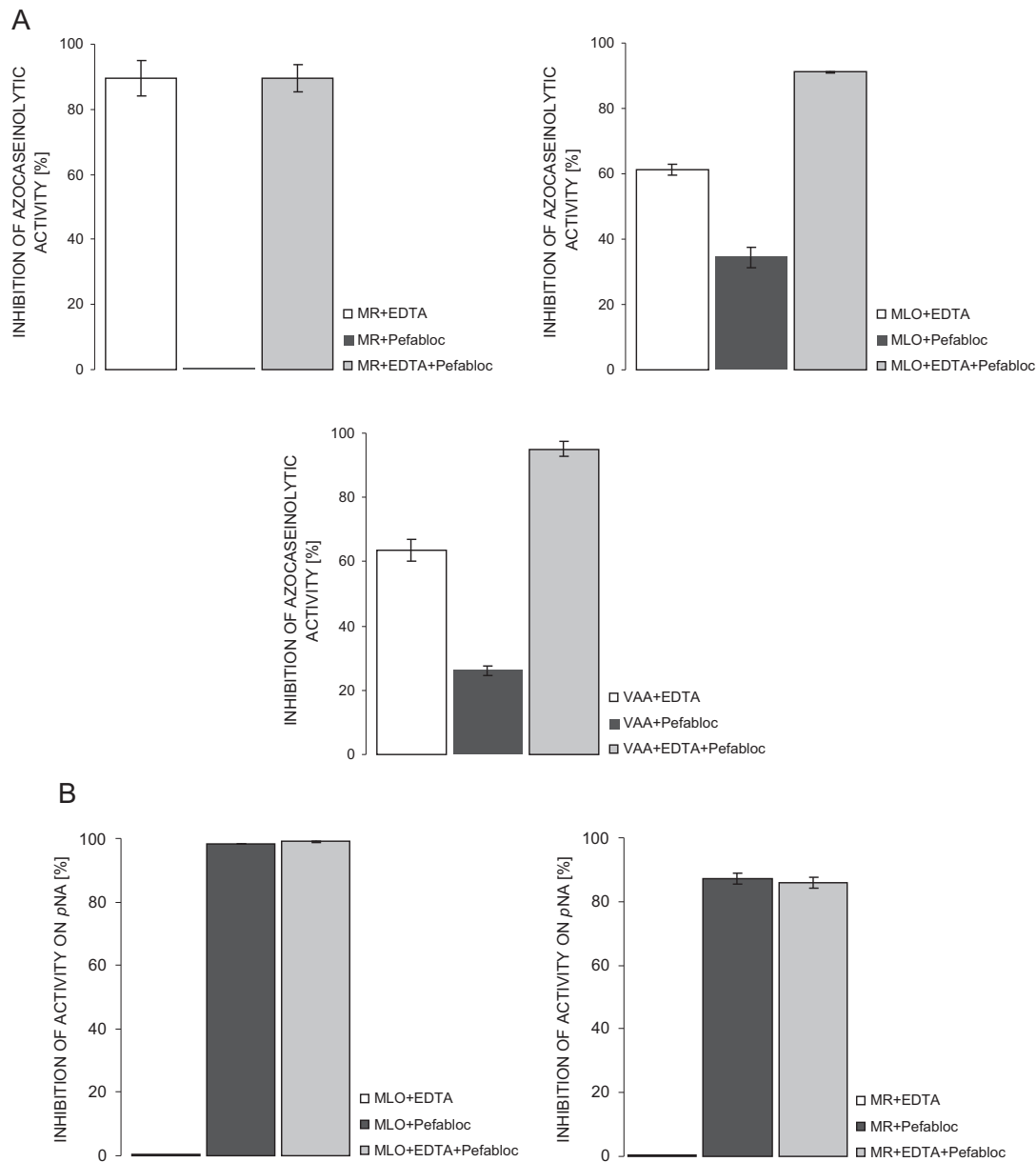
Fig. 2. Proteolytic activity of *Mo. raddei* (MR), *Ma. l. obtusa* (MLO) and *V. a. ammodytes* (VAA) venoms. The resulting colour of the proteolytic reaction was measured spectrophotometrically and plotted against the incubation time. The slope of the line represents the velocity of the enzymatic reaction and was used as a measure of proteolytic activity. Proteolytic activity of untreated venom was considered as 100% and results obtained for Na_2EDTA- or Pefabloc-inhibited venom were expressed in relation to it. (A) Proteolytic activity measured on azocasein. (B) Proteolytic activity measured on N_α-benzoyl-L-Phe-L-Val-L-Arg-p-nitroanilide (pNA). All tests were performed two times and results are given as mean ± SE.

against proteolytic activity of homologues venom, abolishing it almost completely, at the same time inhibiting on average just 10 and 20% of azocaseinolysis by *Mo. raddei* and *Ma. l. obtusa* venom, respectively (Fig. 3A). In order to eliminate serine proteinase activity and thus increase the sensitivity of the biochemical assay by focusing on Zn^{2+}-dependent metalloproteinases only, another set of neutralisation experiments was performed with Pefabloc-inhibited venoms, again on azocasein. Under such conditions, the inhibiting efficacy of anti-venom serum basically remained unchanged – the results obtained on venoms without active serine proteinases were comparable to those of their Pefabloc-untreated versions (Fig. 3B). Neutralisation ability of anti-venom serum was additionally

examined on N_α-benzoyl-L-Phe-L-Val-L-Arg-p-nitroanilide as well. Almost fully efficiency was achieved only on control, homologous venom (60 μg) (Fig. 3C). The partial inhibition of amidolytic activity of around 60% was observed with *Mo. raddei* venom (300 μg), while the neutralisation effect completely failed when *Ma. l. obtusa* venom (60 μg) was used as a proteolytic agent.

3.3. Immunological similarity of Mo. raddei and Ma. l. obtusa venoms with V. a. ammodytes venom

Immunological profile of venoms was investigated by fractionation of the proteins by 2D gel-electrophoresis and their subsequent submission to Western blotting
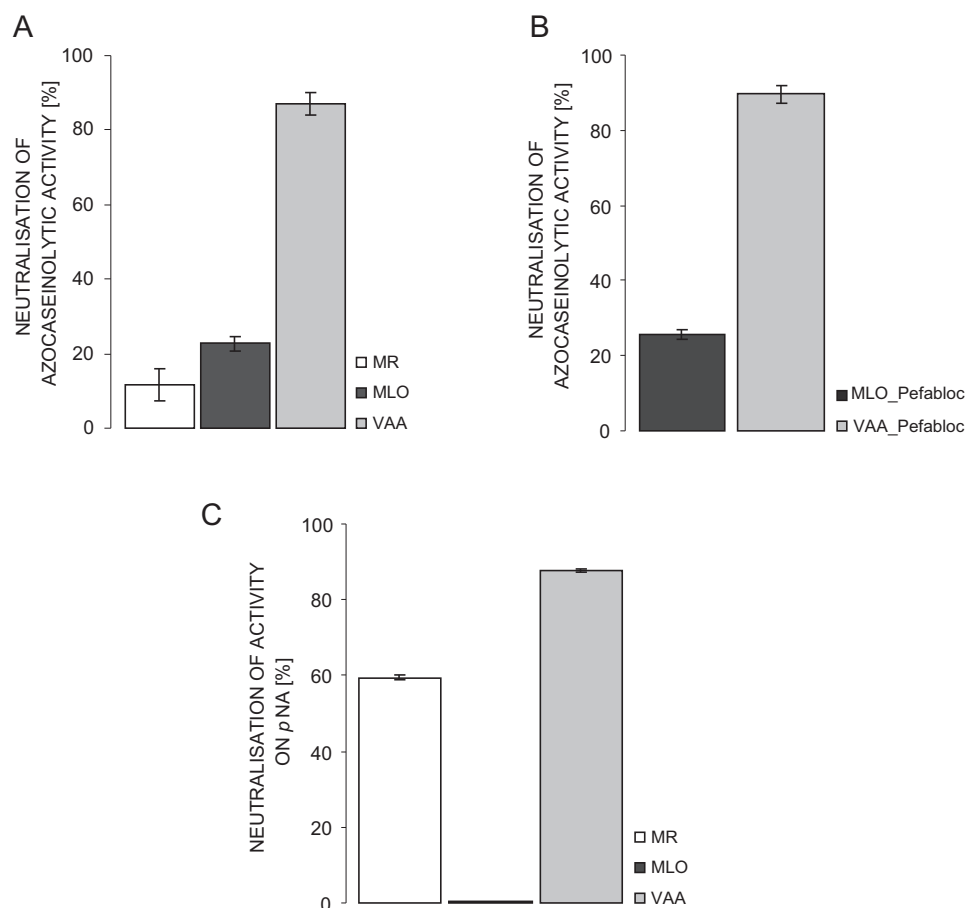
Fig. 3. Proteolytic activity neutralisation measurement. (A) *Mo. raddei* (MR), *Ma. l. obtusa* (MLO) or *V. a. ammodytes* (VAA) venom was preincubated either with anti-venom serum or with serum of non-immunised rabbit, both tested in four-fold dilution, using azocasein as a substrate. (B) Pefabloc-inhibited MR or MLO venom was preincubated either with anti-venom serum or with serum of non-immunised rabbit, both tested in four-fold dilution, using azocasein as a substrate. (C) MR, MLO or VAA venom was preincubated either with anti-venom serum or with serum of non-immunised rabbit, both tested in five-fold dilution, using N_α-benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide (*p*NA) as a substrate. Results are given as mean ± SE of three independent determinations.

using Zagreb antivenom. According to the results obtained by 2D analysis, those two venoms are compositionally clearly distinguishable from that of *V. a. ammodytes*. 2D electropherogram regions indicating the most pronounced qualitative differences in their proteomes are indicated in Fig. 4A. Furthermore, as it can be concluded from the immunoblotting results, Zagreb antivenom shows strong cross-reactivity towards components of *Mo. raddei* venom, which suggests the significant level of their immunological similarity (Fig. 4B). The most obvious 2D spots lacking immunorecognition with antibodies from Zagreb antivenom are 15 kDa proteins focusing either in acidic or neutral to slightly basic range, as well as those with pI of approximately 9–10 and molecular mass between 10 and 60 kDa. On the contrary, the antivenom recognition of proteins constituting *Ma. l. obtusa* venom was proved somewhat poorer. The major non- or weakly immunoreacting components are proteins of molecular mass between 15 and 20 kDa, exhibiting isoelectric point of around 3, than acidic representatives of 25 kDa, as well as those focusing in neutral to slightly basic range of approximately 20 and 40 kDa. The non-recognised portion of *Ma. l. obtusa* venom also belongs to 10, 12.5 and above 20 kDa constituents with isolectric point between 9 and 10.

4. Discussion

Establishment of the clinical application range of novel or existing antivenoms may be guided by knowledge of their immune cross-reactivity towards heterologous venoms, thus possibly circumventing the restricted availability of species-specific immunotherapy for the management of snake bite envenomings in some regions (Calvete et al., 2009; Calvete, 2010). In order to assess the degree of paraspecificity of antivenom produced against the venom of *V. a. ammodytes* (Zagreb antivenom) towards the venoms of two medically important Armenian snakes, *Mo. raddei* and *Ma. l. obtusa*, assays of neutralisation of their relevant biological activities, implemented with biochemical methods focused on inhibition of different proteolytic activities they exhibit, were performed.

Zagreb antivenom was effective in the paraspecific neutralisation of both lethal toxicity and hemorrhagic activity of investigated venoms, thus reflecting satisfactory degree of immunorecognition of their major toxic constituents which are probably antigenically highly conserved despite geographical distance of the species included in the study. Expectedly, antivenom's protective ability against homologous venom was higher than that achieved for *Mo. raddei* and *Ma. l. obtusa* venoms, probably because *V. a.*
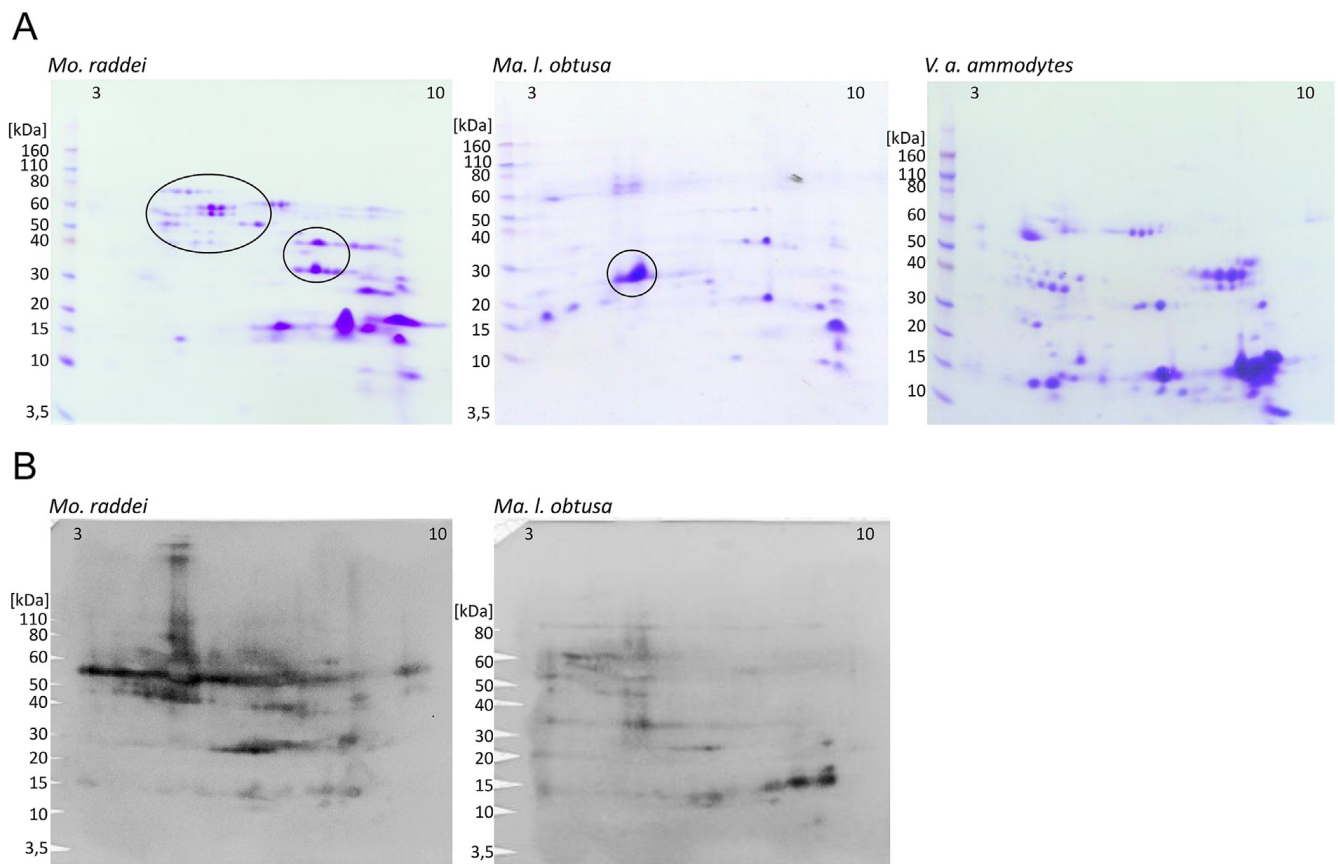
Fig. 4. 2D SDS-PAGE analysis and cross-reactivity of *Mo. raddei* and *Ma. l. obtusa* venoms by Zagreb antivenom. The venoms (250 μg) were applied to IPG strips (non-linear pH 3–10) that were reduced and alkylated prior their loading to 4–12% Bis–Tris precast gels with MES as running buffer under reducing conditions. Gels were stained with acidic Coomassie Brilliant Blue R250 solution (A) or submitted to electro-blotting and incubation with Zagreb antivenom (1 000 000-fold diluted) (B). 2D electropherogram gel regions indicating the most pronounced qualitative differences in the proteomes of *Mo. raddei* and *Ma. l. obtusa* in respect to that of *V. a. ammodytes* are indicated by circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ammodytes-specific F(ab′)₂ fragments have lower avidity for heterologous proteins than for the antigens from the immunisation mixture used for their production. Additionally, paraspecific protective power towards analysed venoms was of comparable degree and largely independent of their lethality and hemorrhagicity. Namely, *Mo. raddei* venom, despite being characterised as twice more lethal, was neutralised with approximately equal or even better capacity than *Ma. l. obtusa* venom. Similar phenomenon was observed by Archundia et al. (2011). On the other hand, in the study performed by Casasola et al. (2009) protection efficacy of equine antiserum specific for the strongly neurotoxic *Naja melanoleuca* venom clearly showed negative correlation with the lethal toxicity of investigated African *Naja* species. According to the authors' explanation, the venoms of stronger toxicity are probably very abundant in neurotoxins, the main cause of death in mice, whose neutralisation with anti-neurotoxin IgGs from experimental serum ensures high rate of survival, while those exhibiting low lethality either lack neurotoxins or contain non-neurotoxic components which are immunologically weakly recognisable by the antiserum. On the contrary, the overall lethal action of *Mo. raddei* and *Ma. l. obtusa* venoms probably is not associated with just one particular or dominantly acting group of toxins whose anti-*V. a.*

ammodytes antivenom-mediated immunocapturing would be suffice to prevent lethality. Additionally, in the case of *Ma. l. obtusa* venom, our results drastically differ from those obtained by Archundia et al. (2011) who tested paraspecificity of experimental trivalent equine antiserum raised against the venoms of *V. a. ammodytes*, *V. aspis* and *V. berus*, reporting complete lack of their immunoreactivity, even at the highest dose, at least when it was assessed by the lethality neutralisation assay in mice. Possible explanation could be that increased polyvalence in antivenoms diminishes their neutralisation potency through interference of some venoms in immunisation mixture with the immune response against others, as warned by Theakston et al. (2003). Additional causes of occasional enhancement or reduction of neutralising capacity of antivenoms could also be associated with variability of either the venoms' composition (at intraspecies level) or the power of production animals' immune response. Furthermore, in our study *Mo. raddei* venom, despite being characterised as at least five times more hemorrhagic, was neutralised with even capacity as the venom of *Ma. l. obtusa*. When the experiment was performed with the venoms adjusted to the same number of MHD doses, slightly stronger protection was achieved against that of *Mo. raddei*, suggesting that Zagreb antivenom shows somewhat better

immunoreactivity towards its hemorragins. Such hypothesis is supported by the results of 2D immunoblotting of targeted venoms probed with Zagreb antivenom. Namely, the cross-reactivity with the components of apparent molecular mass between 50 and 60 kDa, the most likely P-III metalloproteinases, undoubtedly was much stronger for those from *Mo. raddei* venom.

The principal clinical signs induced by *Mo. raddei* and *Ma. l. obtusa* envenomation probably can be predominantly attributed to the proteolytic action of snake venom Zn^{2+}-dependent metalloproteinases (SVMPs) and serine proteinases (SVSPs). Functionally, SVMPs dominantly exert hemorrhage, but also play a relevant role in other local effects, including necrosis and blistering, as well as apoptosis and inflammatory response related to induction of oedema (Rucavado et al., 2004; Gutiérrez et al., 2005), while SVSPs are mostly responsible for provoking systemic hemodynamic disturbances (Serrano and Maroun, 2005). Proteomic analysis of the venoms of Armenian mountain snakes allowed identification of members from both protein families, at the same time confirming metalloproteinases and serine proteinases as the only types of proteolytically active enzymes (Sanz et al., 2008). According to the obtained results, rabbit serum specific for *V. a. ammodytes* venom exhibited varying potential to neutralise azocaseinolytic and amidolytic activities of *Mo. raddei* and *Ma. l. obtusa* venom fractions with the imminent pathophysiological role. Since *Mo. raddei* venom is completely devoid of serine proteinases active on azocasein, quenching of its proteolytic potential, although negligible, probably stems from *in vitro* paraspecific neutralisation of SVMPs solely. Likewise, slightly higher efficiency in abrogation of azocaseinolysis mediated by *Ma. l. obtusa* venom, according to the most likely scenario, also is due to cross immunorecognition of SVMPs, since elimination of SVSPs with Pefabloc treatment did not improve neutralisation ability of anti-venom serum at all. Briefly, although protective efficacy of *V. a. ammodytes*-specific commercial antivenom against hemorrhagicity of both tested venoms was only somewhat weaker than that against the homologous one, inhibiting potential of its analogue, experimentally produced rabbit serum, towards their SVMPs was proved surprisingly low when assessed *in vitro*. Similar effect was observed by Gutiérrez et al. (2013) who studied preclinical efficacy of anti-*Bothrops asper*, *Crotalus simus* and *Lachesis stenophrys* antivenom towards venoms of the snakes from 'Porthidium group' and concluded that neutralisation of their biological activities principally occurs at lower antivenom/venom ratios than that required for abrogating the enzymatic ones. Possible explanation for observed discrepancy between *in vivo* and *in vitro* efficacy of serum specific for *V. a. ammodytes* venom is probably associated with the fact that the most potent hemorrhagins generally belong to the high molecular mass SVMPs from P-III group (Fox and Serrano, 2005), while the proteolytic activity on azocasein is predominantly due to small P-I metalloproteinases that are only weakly if at all hemorrhagic (Bernardes et al., 2008; Gomes et al., 2009, 2011). So, as confirmed by *in vivo* neutralisation assay, IgGs produced by immunisation with *V. a. ammodytes* venom probably have satisfactory immunoreactivity towards P-III members of

Mo. raddei and *Ma. l. obtusa* venoms, but show the overall lower recognition of their SVMPs classified into P-I group. In both venoms included in the study serine proteinases were the only representatives proteolytically active on N_α-benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide. Anti-venom serum partially inhibited those from *Mo. raddei* venom, at the same time being completely ineffective towards *Ma. l. obtusa* SVSPs, corroborating the result obtained on azocasein. Since SVSPs are often associated with life threatening coagulation disturbances, it would be relevant to investigate the possible pathophysiological relevance of these toxins and, additionally, to assess the ability of Zagreb antivenom to neutralise coagulant activity of *Mo. raddei* and *Ma. l. obtusa* venoms *in vivo* as well.

In conclusion, Zagreb antivenom used for treating snake bites in Europe is effective, at the preclinical level, in the neutralisation of lethality and hemorrhagicity of the venom of *Mo. raddei*. It ensures the protective efficacy comparable to that which has been accomplished against *Ma. l. obtusa* venom, either in this study or during routine regulatory testing of the antivenom, and has potential for achieving clinical coverage for envenomings induced by both Armenian mountain snakes. Results encourage the clinical studies planning to provide necessary final proof on clinical effectiveness of anti-*V. a. ammodytes* antivenom in the treatment of envenomings caused by *Mo. raddei* and *Ma. l. obtusa* in Armenia.

Conflict of interest

Maja Lang Balija is the employee of Institute of Immunology Inc., the producer of Zagreb antivenom used in this study.

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