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Vipera ammodytes bites treated with antivenom ViperaTAb: a case series with pharmacokinetic evaluation

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ABSTRACT
Context: In clinical practice it is difficult to differentiate between V. berus and V. ammodytes venomous bites. In the past this was not a concern, but due to the current shortage in Viperfav™ and European viper venom antiserum availability, V. a. ammodytes venomous bites have recently been treated with ViperaTAb®, which is a pharmaceutical formulation containing a monospecific ovine Fab fragments against the venom of V. berus.

Objective: To evaluate ViperaTAb® in V. a. ammodytes envenomations.

Materials and methods: This is a prospective case series of three consecutive patients envenomed by V. a. ammodytes snakebite treated with ViperaTAb®. V. ammodytes venom, neurotoxic ammodytoxins, and Fab fragment levels were determined in serum samples and a pharmacokinetic analysis of the antivenom Fab fragments was carried out.

Results: Three patients bitten by V. a. ammodytes with extensive local swelling, neurological symptoms and recurrent thrombocytopenia were treated with ViperaTAb®. V. ammodytes venom was detected in serum of all three patients. Ammodytoxins were detected in the serum of only the most severely envenomed patient who developed neurological symptoms. In the presented moderate cases, a dose of 8 mL of ViperaTAb® reduced swelling and improved systemic effects, such as thrombocytopenia. However, this dose of ViperaTAb® was not effective in the most severely envenomed patient with the highest serum values of V. ammodytes venom. In this case ViperaTAb® did not stop local swelling and it had no effect on neurological signs. ViperaTAb®s systemic clearance, distribution and elimination half-lives were 4.3–13.4 mL/h/kg, 1.2–3.2 h and 14.1–55.4 h, respectively.

Conclusions: In patients envenomed by V. a. ammodytes venom, ViperaTAb® reduces moderate swelling and temporarily improves systemic effects, except neurological symptoms. ViperaTAb® application induces a decrement of V. ammodytes venom level in the blood, but did not affect serum concentration of neurotoxic ammodytoxins in the one patient with measurable concentrations.

Introduction
In Slovenia Vipera b. berus (Vbb) and Vipera a. ammodytes (Vaa) are the only medically important venomous snakes [1–4]. In clinical practice it is difficult to differentiate between Vbb and Vaa venomous bites. However, in the 15% of cases with neurological signs, a clinical diagnosis of Vaa bite can be made since ammodytoxins (Atxs), the main neurotoxins of Vaa venom, are not present in Vbb venom [4]. Local and systemic symptoms are usually more pronounced in the cases of Vaa envenomation; however, the clinical picture may depend on the patient’s age, body mass, co-morbidities, site of the bite, and amount of injected venom. Accordingly, the differentiation between Vbb and Vaa venom envenoming based on clinical presentation is very difficult and unreliable.

In the past this was not a concern, since Vbb and Vaa snakebites were successfully treated with Viperfav™ (Aventis Pasteur, MSD, Lyon, France), a formulation containing polivalent equine F(ab’)2 fragments as an active principle against venoms of V. aspis, V. berus and V. ammodytes [5], or European viper venom antiserum (Zagreb antivenom) (Institute of Immunology Inc., Zagreb, Croatia), a formulation containing monospecific equine F(ab’)2 against V. ammodytes venom [2,3]. European viper venom antiserum was clinically effective also in paraspecific neutralisation of several venoms from viperid snakes closely related to Vaa – V. aspis, V. berus, V. xanthina and V. lebetina. However, due to a current shortage in Viperfav™ and European viper venom antiserum availability, Vbb and Vaa venomous bites have recently been treated with ViperaTAb® (MicroPharm Limited, Newcastle Emlyn, UK), which is a pharmaceutical formulation containing monospecific ovine Fab fragments against the venom of V. berus [6].

The use of monospecific V. berus antivenom in Vaa bites might be insufficient since it was recently shown that the proteome of Vbb venom is much less complex than the...
venom of Vaa. In particular, it contains lower levels of snake c-type lectin-like proteins (snaclecs) and no Atxs, neurotoxic-secreted phospholipases A$_2$ (sPLA$_2$) [4]. Atxs are responsible for the most characteristic feature of Vaa venom envenoming—induction of neurotoxic signs in patients, while snaclecs are probably responsible for thromboctopenia [4]. However, in vitro immunological experiments revealed that the anti-V. berus antivenom ViperaTAb$^\circledR$ exhibits substantial cross-reactivity with the venom of other Vipera snake species, including V. ammodytes [6]. In addition, in vivo preclinical efficacy studies demonstrated that ViperaTAb$^\circledR$ effectively neutralises lethality induced by V. berus and V. ammodytes and at much higher levels than those outlined by regulatory pharmacopeial guidelines and by the other antivenoms [6]. However, there is no clinical report of monospecific V. berus antivenom usage in the patient bitten by Vaa.

In this case series we describe patients envenomed by Vaa snakebite treated with ViperaTAb$^\circledR$ and pharmacokinetics of the antivenom Fab fragments.

Materials and methods

Patients

This is a prospective case series of consecutive patients envenomed by Vaa snakebite and treated with ViperaTAb$^\circledR$ at the Emergency Department and Centre for Clinical Toxicology and Pharmacology at the government University Medical Centre Ljubljana in 2015. The hospital is a tertiary referral center in the Slovenian capital city of Ljubljana, serving a local population of 400,000 inhabitants and a national population of two million.

The study was approved by the Slovenian National Medical Ethics Committee (No. 87/07/15).

Reagents and chemicals

Horseradish peroxidase-conjugated rabbit anti-guinea pig IgG (HRP-anti-guinea pig IgG), horseradish peroxidase-conjugated rabbit anti-equine IgG (HRP-anti-equine IgG) and horseradish peroxidase-conjugated rabbit anti-equine F(ab')$_2$ (HRP-anti-equine F(ab')$_2$) were from Bio-Rad Laboratories, USA. Horseradish peroxidase-conjugated rabbit anti-ovine IgG (HRP-anti-ovine IgG) was from antibodies-online.com, USA. Bovine serum albumin (BSA), Tween 20 and o-phenylenediamine dihydrochloride (OPD) were from Sigma-Aldrich, USA. Chemicals for buffers and solutions were from Kemika, Croatia.

Snake venoms and antivenoms

Venoms were collected by manual method of milking snakes. Snakes (V. ammodytes ammodytes) were collected in different geographical regions of Croatia and kept in serpentarium (Institute of Immunology, Inc., Zagreb, Croatia) for several years according to good manufacturing and veterinary practice. Approximately, 100 snakes, both male and female, over 3-year-old, per one venom batch were used. Immediately after milking, venom batch was dried in vacuum desiccator with a silica gel at room temperature. After venom crystallisation, obtained venom was kept in dark bottle and stored in the dark at 2–8°C until used. All venom batches were tested for biochemical properties (protein content and analysis by SDS-PAGE and HPLC) and biological activity (lethal dose (median lethal dose, LD$_{50}$), haemorrhagic (minimum haemorrhagic dose – MHD) and necrotic (minimum necrotic dose – MND) activity) [7–9]. Recombinant AtxA used as standard in ELISA assay was produced as described [10]. ViperaTAb$^\circledR$ was supplied by MicroPharm Ltd., Newcastle Emlyn, UK. European viper venom antiserum was from Institute of Immunology Inc., Croatia.

Determination of Vaa venom in sera samples

Microtiter plate was coated with in-house rabbit anti-Vaa venom IgG (1 µg/mL) in 0.05 M carbonate buffer, pH 9.6 (100 µL/well) and left overnight at room temperature (RT). After washing and blocking with 2% (v/v) BSA in PBS buffer comprising 0.05% (v/v) Tween 20 (200 µL/well) for 2 h at 37°C, the investigated sera (2 or 10-fold diluted, depending on the patient) were added in duplicates and incubated overnight at RT.

The whole venom solution (100 ng/mL) used as a standard was prepared in the respective matrix and added in eight serial 2-fold dilutions in duplicates (100 µL/well). Pool of sera from non-bitten individuals (2 or 10-fold diluted) was used as negative control. The plate was extensively washed and incubated first with in-house equine anti-Vaa venom IgG (100 µL/well of 5.7 µg/mL) and then with HRP-anti-equine IgG (100 µL/well of 4000-fold dilution). Finally, after washing, ODP solution (5.5 mM in 0.15 M citrate-phosphate buffer, pH 5.0) with 30% H$_2$O$_2$ (0.5 µL/mL of ODP solution) was added and incubated for half an hour at RT in the dark. The enzymatic reaction was stopped with 1 M H$_2$SO$_4$ (50 µL/well) and absorbance at 492 nm was measured. Venom content was determined by multiplying each concentration, which was obtained from the standard curve, by the corresponding dilution factor. The assay was performed independently minimally two times and the results are given as mean ±95% confidence interval (CI). Pharmacokinetic parameters were determined as described [5].

Determination of Atxs in sera samples

ELISA for determination of Atxs was performed in a similar manner with few exceptions. Coating was done with in-house rabbit anti-Atx IgG (1 µg/mL, 100 µL/well). The investigated sera (2 or 4-fold diluted) and pure Atx solution as standard (eight serial 2-fold dilutions starting from 10 ng/mL) were added after washing and blocking. After washing, plates were incubated first with in-house guinea pig anti-Atx IgG (100 µL/well of 0.3 µg/mL) and then with HRP-anti-guinea pig IgG (100 µL/well of 10,000-fold dilution). The final steps were performed as already described in previous section.
Quantification of antivenom in sera samples

ELISA for determination of antivenom IgG fragments – Fab or F(ab')2 was performed as follows. Microtiter plate was coated with Vaa venom (1 μg/mL) in 0.05 M carbonate buffer, pH 9.6 (100 μL/well) and left overnight at RT. After washing and blocking with 2% (v/v) BSA in PBS buffer comprising 0.05% (v/v) Tween 20 (200 μL/well) for 2 h at 37°C, the investigated sera were added in a few suitable dilutions in duplicates (100 μL/well). ViperaTAb® (100 ng/mL) or European viper venom antiserum (125 ng/mL) used as standards for Fab and F(ab')2 fragments quantification, respectively, were added in eight serial 2-fold dilutions (100 μL/well), also in duplicates. Incubation was performed overnight at RT. The plate was extensively washed and incubated with HRP-anti-ovine IgG (100 μL/well of 5000-fold dilution) for Fab or HRP-anti-equine F(ab')2 (100 μL/well of 25,000-fold dilution) for F(ab')2 fragments quantification. The final steps were performed as already described in previous section. The assay was performed independently three times and the results are given as mean ±95% CI.

Pharmacokinetic analysis

Pharmacokinetic analysis of the measured concentrations was performed using PKSolver add-in software (version 2.0, China Pharmaceutical University, Nanjing, China) for Microsoft Excel [11]. Concentration-time data was fitted either to one-, two- or three-compartment model. Akaike information criterion (AIC) and Schwarz criteria (SC) were used for comparison of goodness of their fit [12].

Results

Case series

Case no. 1

A 60-year-old man with arterial hypertension without therapy and with a history of alcohol abuse was working in a remote vineyard in the southern region of Slovenia when he was bitten in the thenar of the right hand by an approximately 50 cm nose-horned viper (Vaa). Immediately after the bite he felt pain and noticed two puncture wounds. Within 10 min his right hand started to swell. Upon arrival to the local physician, approximately one hour after the viper bite, the patient was faint, dizzy, confused, tachycardic (100/min) and hypotensive (85/50 mmHg), with oedema of the affected hand, extending up to a fifth of his forearm toward the elbow. He was vomiting and had syncpe. He was given antiemetic thiethylperazine and infusion of 0.9% NaCl (1000 mL).

The patient was immediately transferred to the University Medical Centre Ljubljana (UMCL) due to a shortage of antivenom. Upon arrival to the Emergency Department (ED) of UMCL, 3 h after the viper bite, the patient was somnolent, tachycardic (100/min), normotensive (110/80 mmHg) and tachypneic (30/min). He felt a strong and intense pain at the envenomation site and the local oedema with erythema extended up to a half of his forearm. However, the remainder of his physical examination was unremarkable and the patient had no neurological symptoms. The initial laboratory tests 3 h after the bite showed leucocytosis (22 × 109/L), thrombocytopenia (26 × 109/L), coagulopathy with prolongation of prothrombin time (0.41), increased D-dimer (10,469 μg/L) and normal fibrinogen level (3.1 g/L). The patient had rhabdomyolysis (myoglobin 1110 μg/L and creatine kinase 7.9 μkat/L) and acute renal failure (creatinine 113 mmol/L). Treatment was continued with isotonic electrolyte composition infusion at the rate 200 mL/h, and 4.5 h after the bite the patient was given 8 mL of ViperaTAb® diluted in 100 mL of 0.9% NaCl within 30 min as the envenomation was graded as 2b according to a modified Audebert’s clinical severity grading [13].

Follow-up studies, 7 h after the bite, revealed significant increase of platelet count (84 × 109/L) and a slight improvement of coagulopathy (prothrombin time 0.48) and rhabdomyolysis (myoglobin 677.6 μg/L). Renal function normalised due to hydration. These transient improvements were followed by profound second thrombocytopenia (18 × 109/L), coagulopathy (prothrombin time 0.24) and extension of local pain, oedema, erythema, lymphangitis, petechiae and ecchymosis up to the upper arm 12 h after the bite. Pseudothrombocytopenia or analytical error due to possible in vitro formation of aggregates within a tube of the first blood sample was excluded by microscopic examination of blood smear and use of different buffers. Direct and indirect anti-platelet antibody tests were negative, as were direct and indirect Coombs tests.

Fourteen hours after the bite, the patient was given a second dose of 8 mL of ViperaTAb® diluted in 100 mL of 0.9% NaCl within 30 min. Platelet number normalised within 2 h (177 × 109/L) again and remained normal afterwards, while coagulopathy significantly improved with prothrombin time reaching 0.53. However, local oedema and erythema extended further to the shoulder and the patient developed neurological signs (bilateral ptosis, ophthalmoplegia and dysphagia) despite treatment with ViperaTAb®.

Eighteen hours after the bite the patient aspirated gastric content due to dysphagia and developed pneumonitis with acute respiratory failure. The patient was sedated with propofol and intubated, and assisted mechanical ventilation and antibiotics were started. Bronchoscopy with pulmonary toilet was done, but immediately afterwards the patient developed extreme arterial hypertension (300/160 mmHg) and treatment with glyceryl trinitrate infusion was started. Blood pressure dropped to 120/60 mmHg within an hour and immediate brain CT scan was normal. This episode with aspiration was followed by a dose of 4 mL of expired Viperfav™ diluted in 100 mL of 0.9% NaCl within 60 min as envenomation was deemed to be grade 3, 21 h after the bite. Viperfav™ effect-}


Subsequent ELISA analysis of serum samples taken 3 h after the bite revealed the Vaa venom level of 160 ng/mL and the Atxs level 9.5 ng/mL. These and subsequent serum concentrations of Vaa venom and Atxs are presented in Figure 1. Antivenoms ViperaTAb\textsuperscript{VR} and Viperfav\textsuperscript{TM} serum concentrations measured by ELISA are presented in Figure 1, while ViperaTAb\textsuperscript{VR} pharmacokinetic data is presented in Table 1.

**Case no. 2**

An 83-year old man with arterial hypertension was bitten in the dorsal side of the right foot in the central part of Figure 1. Analysis of the serum of the patient #1 bitten by Vaa and treated with ViperaTAb\textsuperscript{VR} and Viperfav\textsuperscript{TM}. Panel (A) displays the concentration of Vaa venom in the serum ($c_v$), platelets count and serum concentrations of ViperaTAb\textsuperscript{VR} ($c_{AV}$) and Viperfav\textsuperscript{TM} ($c_{AV}$). Panel (B) shows serum concentrations of Atxs ($c_{Atxs}$), ViperaTAb\textsuperscript{VR} ($c_{AV}$) and Viperfav\textsuperscript{TM} ($c_{AV}$). Legend: black arrow – ViperaTAb application; white arrow – Viperfav application.

**Table 1.** Pharmacokinetic parameters of monospecific ovine Fab fragments (ViperaTAb\textsuperscript{VR}) in the patients envenomed by Vaa after i.V. administration.

<table>
<thead>
<tr>
<th>Case</th>
<th>$t_{1/2a}$ (h)</th>
<th>$t_{1/2b}$ (h)</th>
<th>$V_{ss}$ (mL/kg)</th>
<th>MRT (h)</th>
<th>AUC$_{0-1}$ (l g h/mL)</th>
<th>AUMC (l g h$^2$/mL)</th>
<th>CL (mL/h/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>3.2</td>
<td>154.5</td>
<td>524.2</td>
<td>45.3</td>
<td>666.8</td>
<td>39,347.1</td>
<td>7.9</td>
</tr>
<tr>
<td>#2</td>
<td>1.6</td>
<td>28.2</td>
<td>118.3</td>
<td>13.0</td>
<td>373.1</td>
<td>7311.0</td>
<td>4.3</td>
</tr>
<tr>
<td>#3</td>
<td>1.2</td>
<td>14.1</td>
<td>52.4</td>
<td>39.1</td>
<td>323.5</td>
<td>4207.5</td>
<td>9.1</td>
</tr>
<tr>
<td>#4</td>
<td>1.2</td>
<td>45.3</td>
<td>198.7</td>
<td>13.4</td>
<td>198.7</td>
<td>7760.0</td>
<td></td>
</tr>
</tbody>
</table>

$t_{1/2a}$: distribution half-life; $t_{1/2b}$: elimination half-life; $V_{ss}$: steady-state volume of distribution; MRT: mean residence time; AUC$_{0-1}$: area under the curve at $t = \infty$; AUMC: area under the first-order moment curve; CL: systemic clearance. We were not able to calculate pharmacokinetic parameters for the first dose of the first case due to lack of data.
Slovenia by a nose-horned viper (\textit{Vaao}). Immediately after
the bite he felt pain and within a few minutes the right
foot started to swell. Upon arrival at the ED of the UMCL
four hours after the viper bite the patient was in extreme
pain, confused and hypertensive (200/100 mmHg). Oedema,
lymphangitis and haematoma were extending up to a third
of the lower leg. The remainder of his physical examination
was unremarkable and the patient had no neurological
symptoms. The initial laboratory tests 3 h after the bite
showed leucocytosis (21 \times 10^9/L), thrombocytopenia
(21 \times 10^9/L), slight rhabdomyolysis (myoglobin 97.6 \mu g/L)
and coagulopathy with increased D-dimer (5428 \mu g/L).
Prothrombin time was at lower normal value (0.70) and
fibrinogen level was normal (3.29 g/L). 5.5 h after the bite
the patient was given 8 mL of ViperaTAb\textsuperscript{V}\textsubscript{R}
diluted in 100 mL of 0.9% NaCl within 30 min as the envenomation
was graded as grade 2b. The extension of local signs stopped
and follow-up studies 7.5 h after the bite revealed an
increase of platelet count (136 \times 10^9/L). Afterwards, pro-
found thrombocytopenia reappeared (18 \times 10^9/L) with nadir
30–70 h after bite. 72 h after the bite the patient was given a
second dose of 8 mL of ViperaTAb\textsuperscript{V}\textsubscript{R} diluted in 100 mL of
0.9% NaCl within 30 min as the envenomation was graded as grade 2b.
The extension of local signs stopped and the follow-up studies
revealed an increase of platelet count (147 \times 10^9/L). The
patient developed no neurological symptoms and was dis-
charged in good condition.

Subsequent ELISA analysis of serum samples taken 4 h
after the bite revealed \textit{Vaao} venom level of 50 ng/mL. The
serum concentrations of the \textit{Vaao} venom are presented in
Figure 2. None of the analysed sera samples for Atxs in this
patient gave absorbance values higher than those obtained
for the simultaneously assayed negative control. Antivenom
ViperaTAb\textsuperscript{V}\textsubscript{R} serum concentrations measured by ELISA are pre-
sented in Figure 2, while ViperaTAb\textsuperscript{V}\textsubscript{R} pharmacokinetic data is
presented in Table 1.

Case no. 3
A 73-year old man was bitten in the left thumb in the south-
ern part of Slovenia by a nose-horned viper (\textit{Vaao}). The
patient killed the snake and took a photo of it. Immediately
afterwards he felt pain and his right arm started to swell and
become erythematous. After 6 h local pain, oedema, ery-
thema and lymphangitis extended up to the elbow and the
patient was admitted to the ED of the UMCL. On admission
the patient was tachycardic (124/min) and normotensive
(150/90 mmHg). The initial laboratory tests 7 h after the bite
showed slight thrombocytopenia (133 \times 10^9/L) and increased
D-dimer (3639 \mu g/L). All other laboratory tests were normal.
14 h after the bite the patient was given 8 mL of ViperaTAb\textsuperscript{V}\textsubscript{R}
diluted in 100 mL of 0.9% NaCl within 30 min as the envenomation
was graded as 2a due to the extension of local signs above the elbow and platelet number further
decreased (113 \times 10^9/L). After the ViperaTAb\textsuperscript{V}\textsubscript{R} application the
extension of local signs stopped and the follow-up studies
revealed an increase of platelet count (147 \times 10^9/L). The
patient developed no neurological symptoms and was dis-
charged in good condition on the third day.

ELISA analysis of serum samples taken 7 h after the bite
revealed the \textit{Vaao} venom level of 40 ng/mL. The serum con-
centrations of \textit{Vaao} venom are presented in Figure 2. Measurable Atxs quantity was not detected in the patient’s serum. Antivenom ViperaTAb\textsuperscript{V}\textsubscript{R} serum concentrations are

Figure 2. Analysis of the serum of the patient #2 bitten by \textit{Vaao} and treated with ViperaTAb\textsuperscript{V}\textsubscript{R}. Serum concentrations of \textit{Vaao} venom (\textit{c}V\textsubscript{a}o) and ViperaTAb\textsuperscript{V}\textsubscript{R} (\textit{c}AV\textsubscript{a}o) were determined using ELISA. Platelet count was also monitored in the samples of serum. Legend: black arrow: ViperaTAb\textsuperscript{V}\textsubscript{R} application.
presented in Figure 3 and ViperaTAb® pharmacokinetic data in Table 1.

**Pharmacokinetics of antivenom level decrement**

Pharmacokinetic parameters were determined as already described [5]. Pharmacokinetic parameters of ViperaTAb® obtained from each of three antivenom treated patients are summarised in Table 1.

**Discussion**

ViperaTAb®, which is a pharmaceutical formulation containing a monospecific ovine Fab fragments against the venom of *V. berus* [6], was used in patients envenomed by *Vaa* for the first time according to the PubMed search. The main reason for this was the current shortage of antivenom against *Vaa*, such as Viperfav™ and European viper venom antiserum that were successfully used in *Vaa* venomous bites in the past [2,5,14].

*Vaa* venom was detected in all three cases in the patients' serum and its concentration correlated with the severity of envenomation. Atxs, the most toxic components of *Vaa* venom purified and characterised so far [15], were detected in only the most severely envenomed patient who developed neurological symptoms. In this case, serum venom level was 160 ng/mL, while Atxs concentration was 9.5 ng/mL, what is 17-times less, since Atxs represents only a small part of *Vaa* venom [16,17]. Accordingly, Atxs were not detected in the other two moderately envenomed patients with serum venom levels of only 50 and 40 ng/mL, since the detection limit for Atxs by ELISA test was about 2 ng/mL. Nevertheless, in all three cases the snakes were identified as *Vaa* by the patients or based on the picture of the dead snake.

It seems that ViperaTAb® is effective in moderate *Vaa* bites, graded as 2a and 2b according to modified Audebert's clinical severity grading [13]. In the presented moderate cases ViperaTAb® reduced swelling and improved systemic effects, such as thrombocytopenia. However, ViperaTAb® was not effective in the case of the most severe envenomation with the highest serum values of the *Vaa* venom (grade 3). In this case ViperaTAb® did not stop the extension of local swelling and it had no effect on neurological signs. We hypothesise that this is due to neurotoxic Atxs, since they are not present in the *V. berus* venom used in ViperaTAb® production [4]. This is important information since neurotoxic effects in *Vaa* bites constitute a medical emergency as they can progress from ptosis to intense muscular weakness [2,3]. In this case prolonged dysphagia due to neurotoxicity of *Vaa* venom may result in life-threatening aspiration pneumonitis. Nevertheless, cranial nerve palsies such as dysphagia with aspiration and acute respiratory failure due to neurotoxicity of *Vaa* venom could be treated symptomatically with airway protection, pulmonary toilet and assisted mechanical ventilation.

Toxicokinetic study revealed that ViperaTAb® application induced immediate *Vaa* venom level decrement, although only temporarily. Namely, in two envenomed patients the venom antigen concentration increased once again, remaining measurable for more than 4 days after the snakebite. On the other hand, Atxs serum concentrations underwent only minor change in the most severely envenomed patient, decreasing from 9.5 to 7.9 ng/mL, irrespective of the consecutive administration of two ViperaTAb® doses. In contrast, Viperfav™ application resulted in a prompt drop of Atxs concentration to a level under the detectable level, but this was followed by Atxs re-appearance in low concentration during the remaining time-course period as well. This support our
clinical observation and hypothesis that neurological symp-
toms in severely envenomed patient bitten by Vaa cannot be
reversed by ViperaTAb<sup>®</sup>, but this can be done only with anti-
venom against Vaa venom containing anti-ammodytoxins
fragments, such as European viper venom antiserum and
Viperfav<sup>TM</sup> [2,14]. However, in this case we were unable to
evaluate Viperfav<sup>TM</sup> effectiveness on neurological symptoms
since the patient was sedated due assisted mechanical venti-
lation at the time of Viperfav<sup>TM</sup> application. Prolonged dys-
phagia in the most severely envenomed patient was probably
due to Atxs re-appearance from tissue depot of unneutralised venom at the site of bite. Dysphagia due to
ischemic or haemorrhagic cerebrovascular event was
excluded by brain CT scan and magnetic resonance imaging.

On the other hand, thrombocytopenia established in all
presented patients bitten by Vaa was successively reversed
by ViperaTAb<sup>®</sup>, since a decrease of platelet count in Vaa
envenomations is probably due to component(s) present in
both Vbb and Vaa venoms [4]. Since no agonists of platelet
aggregation have been detected in the venoms, snaclecs
may have been responsible for the occurrence of thrombo-
cytopenia in vivo by activating platelets through the inter-
action with platelet GPIb receptor or mediating platelet
adhesion to blood vessel walls [4]. Accordingly, the signifi-
cant and prompt post-treatment increase in the platelets
count observed within a few hours after ViperaTAb<sup>®</sup>
antivenom administration in these patients could be secondary
to deadhesion or disaggregation of venom-aggregated plate-
lets. Similar effectiveness in reversing thrombocytopenia has
been reported for crotaline-specific antivenom, where the
Fab binding to venom proteins responsible for aggregation
and rapid subsequent release of platelets has been reason-
ably supposed as the most probable scenario creditable for
normalisation of their count [18,19].

The increase of the platelet count after ViperaTAb<sup>®</sup>
application was however only transient and it correlated with a
temporary decrease in serum venom level due to ViperaTAb<sup>®</sup>
application (Figures 1–3). This might be due to low level of
anti-snaclecs Fab in ViperaTAb<sup>®</sup>, since ViperaTAb<sup>®</sup> is pro-
duced by using V. berus venom, where snaclecs are only
scarcely present, while they are abundant in the Vaa venom
[4]. Furthermore, recurrent thrombocytopenia within a few
hours after ViperaTAb<sup>®</sup> application correlated well with Vaa
venom reappearance in serum. Similar recrudescence of
thrombocytopenia was shown due to renewed venom anti-
genemia after clearance of Fab antivenom (CroTAb) in a
woman bitten by a Western diamondback rattlesnake [16].
According to the presented cases thrombocytopenia in Vaa
bitten patients correlates with serum venom level that
depends on Viperfab<sup>®</sup>’s pharmacokinetic parameters such as
systemic clearance of 4.3–13.4 mL/h/kg and distribution and
elimination half-lives 1.2–3.2 h and 14.1–55.4 h, respect-
ively (Table 1). As expected, Viperfab<sup>®</sup>’s pharmacokinetic
parameters are less favourable compared to equine F(ab’)<sub>2</sub>
fragments (Viperfav<sup>TM</sup>) that have systemic clearance of only
1.64 mL/h/kg and distribution and elimination half-lives 7 h
and 4 days, respectively [5]. This heterogeneity in pharma-
cookinetics of different types of neutralising molecules has evi-
dent potential pharmacodynamic implications since high
tissue depot of unneutralised venom, which continuously
keeps being absorbed from the inoculation site into circula-
tion, creates conditions for recurrence phenomena, mostly
local or coagulopathic, if free plasma antivenom is exhausted
prematurely [20].

In this study heterogeneity in Viperfab<sup>®</sup> pharmacokinet-
ics between different subjects was observed as well, since it
depends on several factors, such as patient’s age, genetic
makeup and co-morbidities, such as alcohol abuse.
Additional limitation of this study is a small number of
included patients and infrequent blood sampling during the
first hours after antivenom application. However, we should
be aware that this study was the result of V. ammodytes anti-
venom shortage and it emphasises the importance of the
specific V. ammodytes antivenom availability.

Conclusions
In V. a. ammodytes bitten patients Viperfab<sup>®</sup>, a monospe-
cific V. berus antivenom with good in vitro and preclinical
cross-reactivity with V. ammodytes venom, reduces moderate
swelling extension and temporarily improves systemic effects,
except neurological symptoms. Viperfab<sup>®</sup> application indu-
ces V. ammodytes venom level decrement, but it does not
affect serum concentration of neurotoxic Atxs. Viperfab<sup>®</sup>
doses in V. a. ammodytes bites should be higher and given
repeatedly despite its maximum 53-h long elimination half-
life. No adverse effects of Viperfab<sup>®</sup> were noted in V. a.
ammodytes bitten patients. In future, specific antivenom
against V. ammodytes should be produced, since full protec-
tion in V. a. ammodytes bitten patients by a monospecific V.
berus antivenom could not be always expected.

Disclosure statement
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