



Venom-Induced Blood Disturbances by Palearctic Viperid Snakes, and Their Relative Neutralization by Antivenoms and Enzyme-Inhibitors

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Palearctic vipers are medically significant snakes in the genera *Daboia*, *Macrovipera*, *Montivipera*, and *Vipera* which occur throughout Europe, Central Asia, Near and Middle East. While the ancestral condition is that of a small-bodied, lowland species, extensive diversification has occurred in body size, and niche specialization. Using 27 venom samples and a panel of *in vitro* coagulation assays, we evaluated the relative coagulotoxic potency of Palearctic viper venoms and compared their neutralization by three antivenoms (Inoserp Europe, VIPERFAV and ViperaTAB) and two metalloprotease inhibitors (prinomastat and DMPS). We show that variation in morphology parallels variation in the Factor X activating procoagulant toxicity, with the three convergent evolutions of larger body sizes (*Daboia* genus, *Macrovipera* genus, and *Vipera ammodytes* uniquely within the *Vipera* genus) were each accompanied by a significant increase in procoagulant potency. In contrast, the two convergent evolutions of high altitude specialization (the *Montivipera* genus and *Vipera latastei* uniquely within the *Vipera* genus) were each accompanied by a shift away from procoagulant action, with the *Montivipera* species being particularly potently anticoagulant. Inoserp Europe and VIPERFAV antivenoms were both effective against a broad range of *Vipera* species, with Inoserp able to neutralize additional species relative to VIPERFAV, reflective of its more complex antivenom immunization mixture. In contrast, ViperaTAB was extremely potent in neutralizing *V. berus* but, reflective of this being a monovalent antivenom, it was not effective against other *Vipera* species. The enzyme inhibitor prinomastat efficiently neutralized the metalloprotease-driven Factor X activation of the procoagulant venoms. In contrast, DMPS (2,3-dimercapto-1-propanesulfonic acid), which as been suggested as another potential treatment option in the absence of antivenom, DMPS failed against all

venoms tested. Overall, our results highlight the evolutionary variations within Palearctic vipers and help to inform clinical management of viper envenomation.

Keywords: venom, antivenom, enzyme inhibition, coagulopathy, snakebite

INTRODUCTION

Snakebite affects millions of people annually, killing over 100,000 and leaving many more with severe permanent injuries (1–3). Snake venom affects all physiological pathways reachable by the bloodstream, with blood coagulation itself a particular target (4). Despite their clinical importance, research into coagulotoxins (toxins that disrupt blood coagulation) has lagged behind other toxin types due to inherent difficulties of working with two enzyme systems (blood and venom) concurrently.

While snakebite in Europe is much less common than in African or Asiatic counties, it is still a potentially deadly medical emergency (5, 6). Common symptoms of envenomations from these vipers include local effects at the bite site such as swelling (edema), necrosis, and compartment syndrome (sometimes requiring fasciotomy), and/or systemic effects such as blood disturbances (coagulotoxicity), neuromuscular paralysis, myotoxicity, and hypotension (7–13). Severe envenomations can lead to amputation and/or death (7–13).

The Palearctic region is dominated by a clade of viperid snakes that emerged 20 million years ago, consisting of the genera *Daboia*, *Macrovipera*, *Montivipera*, and *Vipera* (Figure 1), with (*Daboia* +

Vipera) being sister to (*Macrovipera* + *Montivipera*) (14). The basal morphology of this clade is small and species occupy lowland areas in arid regions. On two convergent occasions, gigantism evolved, once in *Daboia* and again independently in *Macrovipera*. Within *Vipera*, *V. ammodytes* is notably larger than other *Vipera* species.

Daboia and *Macrovipera* are well-characterised as causing potent procoagulant toxicity by converting the zymogen Factor X into the activated enzyme form FXa, which in turn converts prothrombin into thrombin, with the endogenous thrombin converting fibrinogen into fibrin, ultimately resulting in the development of well-ordered fibrin clots (15). The toxin class responsible for the coagulopathy produced by these snakes is a derived type of snake venom metalloprotease (P-IIIId SVMP) that is characterised by two lectin toxins covalently linked to each other to form a dimer, with this dimer in turn covalently linked to the metalloprotease enzyme (16–18). In prey animals, this results in subjugation by thromboembolic stroke-induction and cardiovascular collapse from pulmonary embolism, but in human patients, the dilution of venom into a much larger blood volume results in formation of microemboli. While individual microemboli are of no clinical consequence, a net incoagulable state with morbidity and mortality results from the

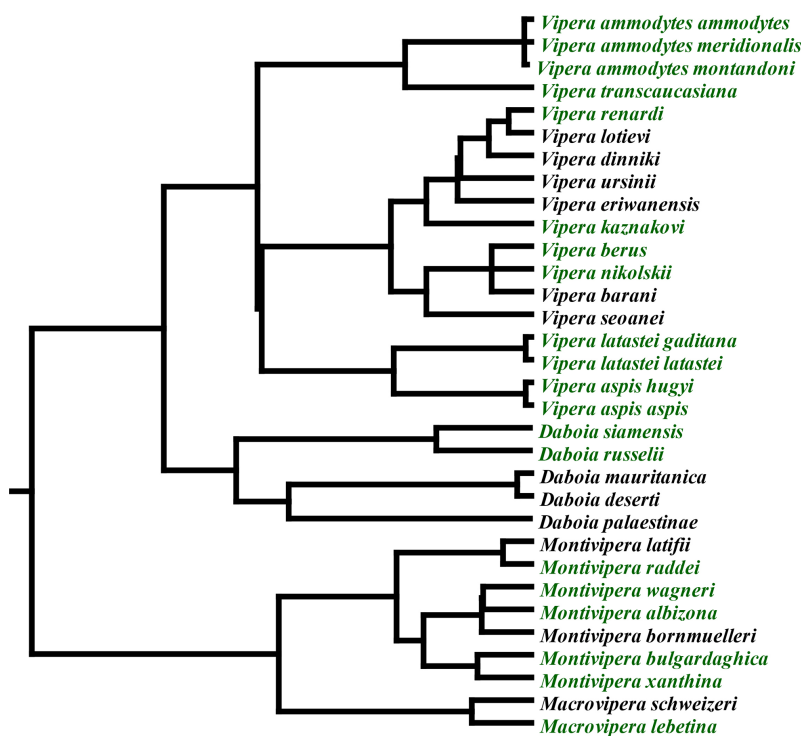


FIGURE 1 | Phylogenetic relationships of Palearctic vipers (14). Green indicates species included in this study.

consumption of clotting factors in the process of venom-induced consumption coagulopathy (19).

The genus *Vipera* is notable for being extremely widespread, radiating across Europe and Central Asia since its emergence 13 million years ago and having complex geographical histories (14, 20–22). Of particular clinical importance for human envenomings is coagulopathy (7–9, 12, 13). Documentation of Factor X activating P-IIIId SVMP in at least one species (*V. ammodytes*) is consistent with its ability to produce severe coagulopathy (23). Sequence alignment shows that *Vipera* shares the characteristic cysteine used for the covalent linkage to the lectin dimer (Figure 2). The presence of FX activating P-IIIId SVMP in *V. ammodytes* venom, like *Daboia* and *Macrovipera* venoms, combined with the observation of FX activation activity in *V. aspis* and *V. berus* venoms (24–26) and the documentation of congruent coagulopathy from *V. berus* envenomations (27) suggests that the presence of FX activating P-IIIId SVMP is the basal state for the *Daboia/ Macrovipera/ Montivipera/ Vipera* clade.

The genus *Montivipera*, endemic to the Near and Middle East, is like *Vipera* in retaining the plesiomorphic small size but is notable for having uniquely specialized for montane habitats (28). The exception is *M. xanthina* which is derived relative to other species within this genus in being larger than the other species and radiating to occupy a lowland niche. Previous work has suggested that unlike the FX activating procoagulant basal state for the clade, this genus also has distinct anticoagulant venoms relative to the rest of the clade (29, 30) and induces

systemic hemorrhage in victims rather than venom-induced consumptive coagulopathy (VICC) (31–34). The mechanism of action for this effect remains to be elucidated.

The divergence of venom documented for the *Daboia/ Macrovipera/Montivipera/Vipera* clade indicates these snakes are an excellent model system to examine the influence of morphology and ecology on pathophysiological venom effects and how this influences clinical treatment options. Furthermore, the development of *in vitro* assays to replace *in vivo* assays to assess antivenom efficacy has been stated as one of the main issues to be addressed for the development and improvement of antivenoms (35). In this study, we tested a wide array of species (Figure 1), ascertained the relative effects upon blood clotting, whether this was mediated by Factor X activation, and the efficacy of three antivenoms and two repurposed enzyme inhibiting drugs. These results not only elucidated the evolutionary processes shaping venom diversification but also how this variation might influence the ability of therapeutics to neutralise pathophysiological effects.

MATERIALS AND METHODS

Stocks Preparation Venoms

All venom work was undertaken under the auspices of UQ IBSC approval #IBC134BSBS2015 and UQ NEWMA approval # 2021/

K9JAWO <i>Daboia russelii</i>	I I L E S G N V N D Y E V V Y P Q K V T A M P K G A V K Q P E Q K Y E D T M Q Y E F E V N G E P V V L H L E K N K I L F
Q7T046 <i>Macrovipera lebetina</i>	I I L E S G N V N D Y E V V Y P Q K I T A L P E A V Q Q P E Q K Y E D T M Q Y E F E V N G E P V V L H L E K N K D L F
MG958498 <i>Vipera ammodytes</i>	I I L E S G N V N D Y E V V Y P Q K I T A L P K G A I Q Q P E Q K Y E D T I Q Y E F E V N G E P V V L H L E K N K G L F
K9JAWO <i>Daboia russelii</i>	S E D Y S E T H Y Y P D G R E I T T N P P V E D H C Y Y H G H I Q N D H S S A S I S A C N G L K G H F K L R G E M Y F
Q7T046 <i>Macrovipera lebetina</i>	S E D Y S E T R Y S P D G R E T T K P P V Q D H C Y Y H G R I Q N D A Y S S A S I S A C N G L K G H F K L Q G E T Y L
MG958498 <i>Vipera ammodytes</i>	S E D Y S E T H Y S P D G R E I T T N P P V E D H C Y Y H G R I Q N D A H S S A S I S A C N G L K G H F L Q G E T Y L
K9JAWO <i>Daboia russelii</i>	I E P L K L S N N E A H A V Y K Y E N I E K E D E T P K M C G V T Q T N W E S D K P I K K A S Q L V S T S A Q -- F N K
Q7T046 <i>Macrovipera lebetina</i>	I E P L K I P D S E A H A V Y K Y E N I E K E D E A P K M C G V T Q T N W E S D E P I K K A S Q L V A T S A K R K F K H
MG958498 <i>Vipera ammodytes</i>	I E P L K I P D S E A H A V Y K Y E N V E K E D E T P K M C G V T E T N W E S D E P I K K A S Q L V A T S E Q Q R F P Q
K9JAWO <i>Daboia russelii</i>	A F I E L I I V D H S M A K K C -- N S T A T N T K I Y E I V N S A N E I F N P L N I H V T L I G V E F W C D R D L I
Q7T046 <i>Macrovipera lebetina</i>	T F I E L V I V D H R V V K K Y -- D S A A T N T K I Y E I V N T V N E I F I P L N I R L T L I G V E F W C N R D L I
MG958498 <i>Vipera ammodytes</i>	R F V E L L I V D L R M V K Y N D S T A I R T R I Y E M V N T V N E I Y I P L N V R V R L I G V E F W C D R D L I
K9JAWO <i>Daboia russelii</i>	N V T S S A D E T L D S F G E W R A S D L M T R K S H D N A L L F T D M R F D L N T L G I T F L A G M C Q A Y R S V G I
Q7T046 <i>Macrovipera lebetina</i>	N V T S S A D T L D S F G E W R G S D L L N R K R H D N A Q L F T D M K F D L S T L G I T F L D G M C Q A Y R S V G I
MG958498 <i>Vipera ammodytes</i>	N V T S S A D T L D S F A E W R G S D L L N R K R H D N A Q L F T D M A F D L N T F G I T F L A G M C Q A Y R S V G I
K9JAWO <i>Daboia russelii</i>	V Q V Q G N R N F K T A V I M A H E L S H N L G M Y H D G K N C I C N D S S C V M S P V L S D Q P S K L F S N C S I H D
Q7T046 <i>Macrovipera lebetina</i>	V Q E H G N K N F K T A V I M A H E L G H N L G M Y H D R K N C I C N D S S C I M S A V L S Q P S K L F S N C S N H D
MG958498 <i>Vipera ammodytes</i>	V K E H -- N I N F K T A V I M A H E L G H N L G M S H D R K N C I C G R S S C I M A P V L S D Q P S K L F S N C S R D Y
K9JAWO <i>Daboia russelii</i>	Y Q R Y L T R Y K P K C I L Y P P L R K D I V S P P V C G N E I W E E G E E C D C G S P A D C Q N P C C D A A T C K L K
Q7T046 <i>Macrovipera lebetina</i>	Y R R Y L T T Y K P K C I L N P P L R K D I A S P P I C G N E I W E E G E E C D C G S P K D C Q N P C C D A A T C K L T
MG958498 <i>Vipera ammodytes</i>	Y Q R Y L T R Y K P K C I L N E P L R K D I A S R A V C G N E I W E E G E E C D C G S P K D C Q N P C C D A A T C K L T
K9JAWO <i>Daboia russelii</i>	P G A E C G N L C C Y Q C K I K T A G T V C R R A R N E C D V P E H C T G Q S A E C P R D Q L Q Q N G Q P C Q N N R G
Q7T046 <i>Macrovipera lebetina</i>	P G A E C G N L C C E K C I K T A G T V C R R A R D E C D V P E H C T G Q S A E C P A D G F H A N G Q P C Q N N R G
MG958498 <i>Vipera ammodytes</i>	P G A E C G N L C C E N C I R T A G T E C R P A R D E C D V P E H C T G Q S A E C P R D Q F Q Q N G Q P C L N N L G
K9JAWO <i>Daboia russelii</i>	Y C Y N G D C P I M R N Q C I S L F G S R A T V A K D S C F Q E N L K G S Y Y G Y C R K E N G R K I P C A P Q D V K C G
Q7T046 <i>Macrovipera lebetina</i>	Y C Y N G D C P I M T K Q C I S L F G S R A T V A E D S C F Q E N Q K G S Y Y G Y C R K E N G R K I P C A P Q D I K C G
MG958498 <i>Vipera ammodytes</i>	Y C Y N G D C P I M T N Q C I S L F G S R A T V A E D S C F E E N L K G S Y Y G Y C R K E N G R K I P C A P Q D I K C G
K9JAWO <i>Daboia russelii</i>	R L F C L N N S P R N K N P C N M H Y S C M D Q H K G M V D P G T K C E D G K V C N N K R Q C V D V N T A Y Q S T T G F
Q7T046 <i>Macrovipera lebetina</i>	R L Y C L D N S P G N K N P C K M H Y R C R D Q H K G M V E P G T K C E D G K V C N N K R Q C V D V N T A Y -----
MG958498 <i>Vipera ammodytes</i>	R L Y C L N N S P G N K N P C N M H Y R C W D Q H K G M V E P G T K C E D G K V C N N K R Q C V D V N T A Y -----

FIGURE 2 | Sequence alignment of representative Factor X activating P-IIIId SVMP characteristic of the Palaearctic viper clade. Highlighted is the diagnostic cysteine that forms the interchain disulfide bond to a covalently linked lectin dimer. Uniprot (*Daboia* and *Macrovipera*) and Genbank (*Vipera*) accession codes are given for each sequence.

AE000075. Pooled venoms (N = 3 captive adults unknown sex) which were immediately snap frozen in liquid nitrogen and kept at -80 until lyophilisation and founder locality (if known) tested were: *Daboia russelii* (Pakistan), *D. siamensis* (Taiwan), *Macrovipera lebetina turanica* (Turkmenistan), *M. schweizeri* (Greece), *Montivipera albizona*, *M. bulgardaghica*, *M. raddei*, *M. wagneri*, and *M. xanthina* (Turkey), *Vipera ammodytes* (Krk Island, Croatia), *V. ammodytes* (Maribor, Slovenia), *V. ammodytes* (Ada Island, Montenegro), *V. ammodytes* (Lake Skadar, Montenegro), *V. ammodytes* (Slunj, Croatia), *V. a. meridionalis* (Greece), *V. a. montandoni* (Bulgaria), *V. aspis aspis* (France), *V. aspis hugyi* (Italy), *V. berus* (Norway), *V. berus* (Slovenia), *V. berus* (Snežnik Mountain, Slovenia), *V. kaznakovi* (Turkey), *V. latastei latastei* (Burgos, Spain), *V. latastei gaditana* (Spain), *V. nikolskii* (Russia), *V. renardi* (Russia), and *V. transcaucasiana* (Turkey). These lyophilized venoms were reconstituted to 1 mg/ml concentrated venom stock (concentration checked using 280 nm wavelength on a Thermo Fisher Scientific™ NanoDrop 2000 UV-Vis Spectrophotometer (ThermoFisher, Sydney, Australia) following the manufacturer's instructions 1 Abs = 1 mg/ml which is recommended when analyzing heterogenous solutions) by adding 50% glycerol and deionized water and stored at -20°C for further use.

Plasma

All plasma work was undertaken under the UQ IBSC approval #IBC134BSBS2015. Two bags of pooled 3.2 % citrated plasma (Label #A540020754341 & #A540020764777) were obtained from the Australian Red Cross (Research agreement #18-03QLD-09 and University of Queensland Human Ethics Committee Approval #2016000256). The two lots of plasma were pooled, aliquoted to 1 ml quantities, flash-frozen in liquid nitrogen, and stored at -80°C until required for testing. Aliquots were defrosted in at 37°C in a Thermo Haake ARCTIC water bath with a SC150-A40 circulator. Plasma aliquots were only used for up to an hour post-defrosting, after-which fresh aliquots were defrosted.

Fibrinogen

To ascertain the effect of venom on human fibrinogen clotting time, 100 mg of fibrinogen (Lot# SLBZ2294 Sigma Aldrich, St. Louis, Missouri, United States) was mixed with Owen Koller (OK) buffer (Stago catalogue #00360) to achieve a concentration of 4 mg/ml, then aliquoted to 1 ml quantities, flash-frozen, and stored at -80°C until further use. Defrosting steps and use were as per 2.1.2.

Antivenom (AV)

Antivenoms tested (and immunizing species for each antivenom) were as follows. Inoserp Europe (lot # 9IT03006), a 22.5 mg/ml F(ab')₂ antivenom made using an immunizing mixture consisting of *Macrovipera lebetina cernovi*, *M. l. obtusa*, *M. l. turanica*, *M. schweizeri*, *Montivipera xanthina*, *Vipera ammodytes*, *V. aspis*, *V. berus*, and *V. latastei*). MicroPharm VIPERFAV (lot #P4A281V), a 100 mg/ml F(ab')₂ antivenom made using an immunizing mixture consisting of *Vipera*

ammodytes, *V. aspis*, *V. berus*). MicroPharm ViperaTAb (lot #VPT 002000), a 24.6 mg/ml Fab antivenom made using *V. berus* as the sole venom in the immunizing mixture. Both VIPERFAV and ViperaTAb came in a concentrate (1 X 4ml and 2 X 4ml vials respectively) solution, while Inoserp Europe was supplied in a lyophilized form, which was reconstituted with 10 ml of deionized water, according to company instructions. All antivenoms were centrifuged (RCF 14000) at 4°C for 10 min, followed by filtration of the supernatant (to remove insoluble material) using 0.45 µm Econofiltr PES (Agilent Technologies, Beijing, China), aliquoted, and then stored at 4°C for future use. For tests (see 2.2.1.2), 5% AV solution was prepared by diluting with Owen Koller (OK) buffer (Stago catalogue #00360) for Inoserp, 4% for ViperaTAb, and 2% VIPERFAV. The percentages were calculated relative to the different antivenom treatment volume (10 ml, 8 ml, and 4 ml respectively) and thus the same proportion of each vial was used for the tests, thus allowing for a vial-to-vial comparison of efficacy. Therefore, calculating the ratios, the above-mentioned percentages were determined.

Enzyme Inhibitors

We set out to determine the efficacy of two small molecule inhibitors prinomastat hydrochloride ((S)-2,2-Dimethyl-4-((p-(4-pyridyloxy) phenyl) sulfonyl) -3- thio- (catalogue# PZ0198) and DMPS 2,3-Dimercaptopropanesulfonic acid sodium salt monohydrate (catalogue # D8016) from Sigma-Aldrich. The powder was first dissolved in 10 % dimethyl sulfoxide (DMSO) and further diluted using deionized water to form 10 mM and 20mM stock solutions, respectively and stored at -80°C.

Assay Conditions

Effects Upon Clotting Times of Plasma and Fibrinogen

Coagulotoxicity Effects on Plasma and Fibrinogen

Determination of venom effects upon coagulation was done using the STA-R Max[®] (Stago, Asnières sur Seine, France) coagulation analyser and adapted from validated coagulation assay protocols (36–39). 1mg/ml venom stocks (50% glycerol/50% deionized water) were diluted to 100 µg/ml with OK Buffer (Stago catalogue #00360) to prepare the working stock, which was later loaded into the analyser for running 8-point concentration curves with serial dilutions of 1, 1/2, 1/5, 1/12.5, 1/30, 1/80, 1/160, and 1/400 (final reaction concentrations of venom). In an automated process, 50 µl venom stock (100 µg/ml starting concentration and serially diluted to form final reaction concentrations as noted above) were added to a cuvette, followed by the addition of 25 µl of OK buffer, 50 µl of 0.025 M calcium chloride (Stago catalogue # 00367), and 50 µl of phospholipid (Stago catalogue #00597), and then the mixture incubated for 2 minutes at 37°C. Subsequently 75µl of plasma or 75µl of 4 mg/ml fibrinogen was added after incubation, and clotting time was recorded immediately. To avoid abnormal results due to venom degradation, venom was changed after each set. As a positive control, coagulation activator kaolin (Stago C-K Prest standard kit, Stago catalogue #00597) was used to check for consistent plasma responses, and 25 µl of

the thrombin (Stago catalogue #115081) as a positive control to check for consistent plasma responses. The negative control for both plasma and fibrinogen studies was 50% glycerol/deionized water that was diluted to the same amount as the venom 50% glycerol venom and positive control stocks (1% final concentration for venom, positive controls, and negative controls).

Vial to Vial Antivenom Efficacy and Enzyme-Inhibitor Efficacy

Efficacy of antivenoms or enzyme inhibitors in neutralizing toxic effects of venom upon blood clotting was tested by repeating the above mentioned 8-point concentration curves, but the 25 μ l of OK buffer (added to the cuvette before incubation) was replaced with 25 μ l of antivenom (final reaction concentration of Inoserp Europe 0.5%, ViperaTAB 0.4%, and VIPERFAV 0.2%). The differences in concentration were reflective of the different antivenom vial size (10 ml, 8 ml, and 4 ml respectively) and thus the same proportion of each vial was used for the tests, thus allowing for a vial-to-vial comparison of efficacy. Similarly, enzyme-inhibitors at 2 mM working stock for prinomastat and DMPS, (final reaction concentration of 0.2 mM) was tested against *Daboia russelii* (Pakistan), *D. siamensis* (Taiwan), *Macrovipera lebetina turanica* (Turkmenistan), *M. schweizeri* (Greece), *V. ammodytes* (Ada Island, Montenegro), *V. aspis hugyi* (Italy).

Thromboelastography

To evaluate the strength of the clot and total thrombus generated by the venoms in plasma, further investigation was carried out by using TEG5000 haemostasis analyzers (Haemonetics®, Haemonetics.com, catalogue # 07- 033). The assay included consecutive addition of 72 μ l of 0.025M CaCl₂, 72 μ l phospholipid, 20 μ l of the OK buffer, 7 μ l of 1 mg/ml of venom, and 189 μ l plasma to the reaction cup, followed by automated measurement. For spontaneous clotting of plasma (negative control), 7 μ l 50% deionized water/glycerol was replaced with venom. Similarly, 7 μ l of thrombin (Stago catalogue #115081 Liquid Fib) or 7 μ l Factor Xa (Stago catalogue #253047 Liquid Anti-Xa) was replaced with venom, for two positive controls. Each reaction ran for 30 minutes.

Clotting Factor Activation Assays

Fluoroskan Ascent™ (Thermo Scientific, Vantaa, Finland) was employed to detect Factor X and prothrombin activation based on the results of the above methods (38, 39). **Table 1** reagents were manually plated in 384-well plates (black, lot#1171125, Nunc™ Thermo Scientific, Rochester, NY, USA), followed by automated pipetting of 70 μ l of buffer containing 5 mM CaCl₂, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.3) and Fluorogenic Peptide Substrate, (ES011Boc-Val-Pro-Arg-AMC. Boc: t-Butyloxycarbonyl; 7-Amino-4-methylcoumarin; R & D systems, Cat# ES011, Minneapolis, Minnesota) in 500:1 ratio to start the reaction; with the plate warmed up at 37°C and shaken for 3 s before each measurement. The reaction was carried out 300 times at 390/460 nm (excitation/emission) and every 10 s the fluorescence generated by the cleavage of the substrate was measured by Ascent® Software v2.6 (Thermo Scientific, Vantaa, Finland). To obtain results, blank (background) values were

TABLE 1 | Fluorescent substrate activation assay.

Blank	20 μ l of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tris-HCl (pH 7.3) + 10 μ l PPL)
Control with Activated Enzyme	10 μ l of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tris-HCl (pH 7.3) + 10 μ l PPL + 10 μ l (10 μ g/ml FXa (Haematologic Technologies catalog # GG0621) or 1 μ g/ml Thrombin (Haematologic Technologies catalog # JJ0701))
Control with Zymogen	10 μ l of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tris-HCl (pH 7.3) + 10 μ l PPL + 10 μ l (10 μ g/ml FX (Haematologic Technologies catalog # HH0821) or 1 μ g/ml prothrombin (Haematologic Technologies catalog # HH1010))
Venom without Zymogen	10 μ l of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tris-HCl (pH 7.3) + 10 μ l PPL + 10 μ l venom
Venom with Zymogen	10 μ l zymogen (10 μ g/ml FX or 1 μ g/ml prothrombin) + 10 μ l PPL + 10 μ l venom (1 μ g/ml FX (Haematologic Technologies catalog # HH0821) or 0.1 μ g/ml prothrombin (Haematologic Technologies catalog # HH1010))

subtracted from all other reactions, followed by subtraction of “venom without zymogen” values from “venom with zymogen” values (to nullify artificial increment of the fluorescence values caused some venoms which work directly on the substrate). Finally, the resultant values from the subtractions were normalized as a percentage relative to FXa or thrombin by organizing in Excel and then analysing in GraphPad PRISM 8.1.1 (GraphPad Prism Inc., La Jolla, CA, USA).

Clotting Factor Inhibition Assays

Samples were diluted at 1:10 with OK buffer. 50 μ l of venom + 50 μ l of CaCl₂ (0.025M, Stago Cat#11851) + 50 μ l of phospholipid solution (STA C.K. Prest standard kit, Stago Cat#12207, solubilized in 5 ml OK buffer) + 25 μ l of clotting factor (thrombin, FIXa, FXa, FXIa, and FXIIa) were incubated for 120s at 37°C. Subsequently 75 μ l of plasma was added, and clotting time was measured. OK buffer control was used as a negative control.

Statistical Analyses

All tests were run in triplicate. All data plotting and statistical analysis were done by using GraphPad PRISM 8.1.1 (GraphPad Prism Inc., La Jolla, CA, USA). Determination of the AV efficacy against venom, the area under the curve (AUC) for both venom and venom + antivenom was calculated using the software, followed by generation of X-fold magnitude of shift in Excel (formulae [(AUC of venom incubated with antivenom/AUC of venom) - 1]). The resulting values for X-fold magnitude of shift; if 0, indicated no neutralization (no change of clotting time curve), and if over 0, demonstrated venom neutralization (change in clotting time curve).

RESULTS

Effects Upon Clotting Times of Plasma and Fibrinogen Coagulotoxicity Effects

The *Vipera* venoms all displayed a clotting effect upon plasma, but with a wide range of values. The maximum velocity clotting times

(in seconds) at the highest concentration (20 µg/ml) of (seconds +/- SD) (with smaller numbers indicating stronger effect) was: 15.4 +/- 0.9 *V. ammodytes* (Lake Skadar, Montenegro); 16.0 +/- 0.1 *V. ammodytes* (Ada Island, Montenegro); 18.4 +/- 1.9 *V. ammodytes* (Maribor, Slovenia); 20.4 +/- 0.5 *V. ammodytes* (Slunj, Croatia); 23.0 +/- 0.6 *V. ammodytes* (Krk Island, Croatia); 23.9 +/- 0.1 *V. berus* (Slovenia); 23.9 +/- 0.1 *V. berus* (Snežnik Mountain, Slovenia); 26.7 +/- 0.3 *V. latastei gaditana* (Spain); 28.7 +/- 0.2 *V. aspis hugyi* (Italy); 30.3 +/- 0.4 *V. a. meridionalis* (Greece); 33.0 +/- 2.4 *V. berus* (Norway); 35.3 +/- 0.4 *V. kaznakovi* (Turkey); 44.1 +/- 0.4 *V. renardi* (Russia); 66.2 +/- 3.4 *V. a. montandoni* (Bulgaria); 98.9 +/- 0.3 *V. aspis aspis* (France); 107.8 +/- 1.4 *V. transcaucasiana* (Turkey); 179.7 +/- 0.3 *V. nikolskii* (Russia); and 359.8 +/- 4.5 *V. latastei latastei* (Spain) (**Figure 3**). The plasma kaolin positive control was 51.0 +/- 0.34, while the negative control (spontaneous clotting) was 645.2 +/- 9.8

To check significant differences the values One Way ANOVA was carried out on the clotting times. There were no significant differences ($p > 0.05$ at 95.00 % confidence interval) between *V. ammodytes* (Krk Island, Croatia) versus (*vs.*), *V. ammodytes* (Slunj), Croatia), *V. berus* (Slovenia), *V. berus* (Snežnik Mountain, Slovenia) and *V. latastei gaditana* (Spain); *V. ammodytes* (Maribor, Slovenia) *vs.* *V. ammodytes* (Ada Island, Montenegro), *V. ammodytes* (Lake Skadar, Montenegro) and *V. ammodytes* (Slunj, Croatia); *V. ammodytes* (Ada Island, Montenegro) *vs.* *V. ammodytes* (Lake Skadar, Montenegro) and *V. ammodytes* (Slunj, Croatia); *V. ammodytes* (Slunj, Croatia) *vs.* *V. berus* (Slovenia), *V. berus* (Snežnik Mountain, Slovenia); *V. a. meridionalis* (Greece) *vs.* *V. aspis hugyi* (Italy), *V. berus* (Norway) and *V. latastei gaditana* (Spain); *V. aspis hugyi* (Italy) *vs.* *V. berus* (Slovenia), *V. berus* (Norway) and *V. latastei gaditana* (Spain); *V. berus* (Norway) *vs.* *V. kaznakovi* (Turkey); *V. berus* (Slovenia) *vs.* *V. berus* (Snežnik Mountain, Slovenia) and *V. latastei gaditana* (Spain); *V. berus* (Snežnik Mountain, Slovenia) *vs.* *V. latastei*

gaditana (Spain), while there were significant difference between clotting times between all other vipers.

However, like shown previously for *Daboia* and *Macrovipera* venoms (38) none of the *Vipera* venoms clotted fibrinogen with the assay measurements reaching the machine maximum of 999 seconds. The clotting time for the thrombin positive control was (seconds +/- SD) 3.6 +/- 0.1 seconds. This suggested that the clotting action shown for plasma was due to the activation of a clotting factor, which was explored further (see 3.3 below).

In contrast, all *Montivipera* venoms demonstrated potent anticoagulant actions on plasma, with the test sets all reaching the machine maximum reading time of 999 seconds. This included the derived *M. xanthina* which has secondarily colonized a lowland habitat relative to the other species in this clade (40) and also evolved a larger body size. Despite these derivations, it retains the potent anticoagulant venom characteristic of the *Montivipera* genus.

Antivenom and Enzyme Inhibitor Efficacy

Inoserp and VIPERFAV were comparable against all *V. ammodytes* populations and *V. a. meridionalis*, while VIPERFAV was moderately less effective for other species, except for *V. latastei gaditana* against which it performed comparatively poorly (**Figures 4 and 5**). Interestingly, both Inoserp and VIPERFAV, which had *V. ammodytes* as their immunizing species, showed lower level potency against *V. ammodytes* species hailing from Montenegro compared to other *V. ammodytes* populations. Consistent with ViperaTAB having only *V. berus* as an immunizing species, it performed extremely well against *V. berus* but compared to the other two antivenoms, it performed poorly against the other species except for moderate levels of neutralization of *V. renardi*.

While the antivenoms had variable differences, prinomastat highly neutralized not only *Vipera* representatives but also *Daboia* and *Macrovipera* (major metalloprotease dependent venoms) representatives at 0.2 mM concentration (**Figure 6**). In contrast, DMPS performed extremely poorly against all venoms (**Figure 6**).

Thromboelastography

Consistent with the activation of a clotting factor and the resulting generation of endogenous thrombin, all venoms produced strong, stable clots in the thromboelastography assays (**Figure 7**).

Clotting Factor Zymogen Activation

All venoms displayed the ability to activate Factor X but only negligible activation of prothrombin (**Figure 8**). The relative potencies were congruent with the action of respective venoms on plasma (**Figures 3 and 7**). *V. ammodytes* (Ada Island, Montenegro), which was the fastest on plasma, showed the highest activation of FX. Conversely, *V. renardi* was the slowest on plasma and also activated FX the least.

Clotting Factor Inhibition

As the *Montivipera* venoms were shown to be potently anticoagulant, tests were undertaken to ascertain if the

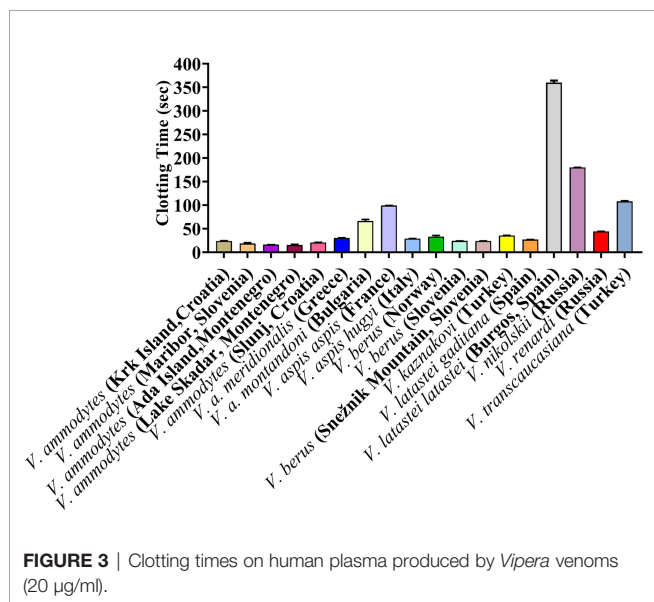


FIGURE 3 | Clotting times on human plasma produced by *Vipera* venoms (20 µg/ml).

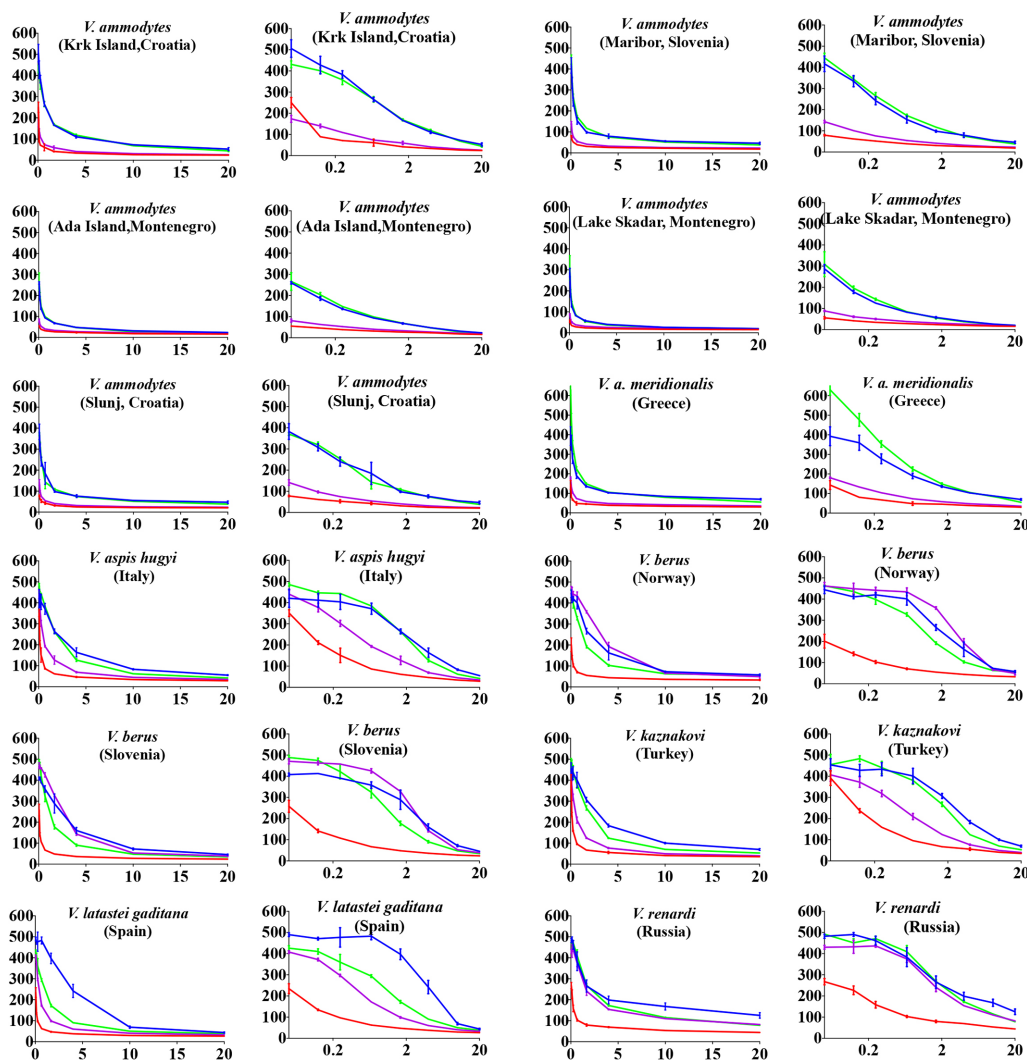


FIGURE 4 | 8-point concentration curves, x-axis showing concentrations of venom in $\mu\text{g/ml}$ and y-axis showing clotting times in seconds of human plasma with venom and relative antivenom efficacy. For each species, linear graphs are presented on the left and logarithmic views on the right. Shown are: venom-induced clotting times (red curves); venom-induced clotting times after preincubation with Inoserp AV (final concentration 0.5%; spontaneous control- 420.2 \pm 27.7) (blue curves), ViperaTab AV (final concentration 0.4%; spontaneous control- 478.4 \pm 33.3) (purple curves), or VIPERFAV AV (final concentration 0.2%; spontaneous control- 509.7 \pm 13.5) (green curves). Values are mean \pm SD of N = 3 and shown as dots with error bars. Some error bars are too small to see.

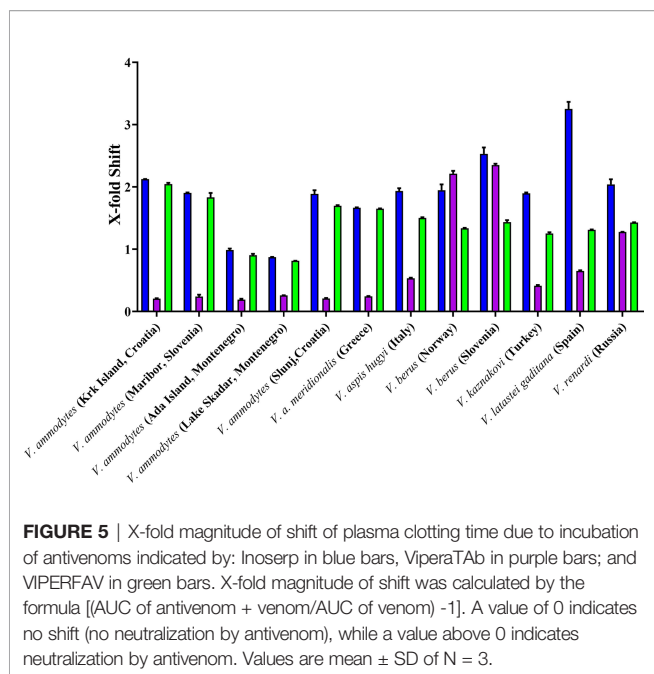
inhibition was due to the inhibition of thrombin, FIXa, FXa, FXIa, or FXIIa. Only FXa was shown to be inhibited, with all the species having this action at comparable potency (**Figure 9**).

DISCUSSION

Our study set out to examine changes in venom biochemistry relative to two distinct types of derivation in this clade away from the diminutive, low-land niche occupying last common ancestor: that of the three convergent evolutions of gigantism (*Daboia* genus, *Macrovipera* genus, and *Vipera ammodytes* as the significantly largest member of the *Vipera* genus); and the

occupation of the high-land niche by the *Montivipera* genus and *Vipera latastei latastei* as a unique high-land specialist within the *Vipera* genus.

Consistent with previous results demonstrating that *Daboia* and *Macrovipera* species have extremely potent procoagulant (Factor X activating) venoms (38), this study revealed that the largest *Vipera* species (*V. ammodytes*) was also the most potent *Vipera* in activating Factor X, with one population (Lake Skadar, Montenegro) even approaching that of *Daboia* and *Montivipera* speed of action. Consistent with the link between size and relative FX activation levels of the venoms, the more diminutive sister species *V. transcaucasiana* and all other smaller *Vipera* species were comparably less potent than *V. ammodytes*. This is in



contrast to other snakes, such as the *Bitis* genus in which gigantism evolved on two separate occasions (41), neither of which were linked to notable changes in coagulotoxicity (42).

The strongest divergence in venom action was for the *Montivipera* species. The diversification into a unique high-altitude niche relative to the low-altitude last common ancestor of the Palearctic viper clade was accompanied by a change from the procoagulant ancestral trait to potent anticoagulant toxicity. Specifically, instead of activating Factor X into Factor Xa like the last common ancestor of the Palearctic viper clade, the venom of these snakes was shown in this study to inhibit Factor Xa. This radical change in venom biochemistry was retained in *M. xanthina*. This is significant as this species is nested deep within the *Montivipera* genus (Figure 1), and has secondarily evolved to occupy a low-land niche from within this montane specialist genus (40). However, the relatively recent shift from high-land to low-land niche has not yet been accompanied by a change in venom biochemistry. The relationship between a secondary losses of the ancestral procoagulant trait accompanying the specialization for a high-land niche was reinforced by the convergent action within *V. latastei*, which has two subspecies: the low-land *V. latastei gaditana* and the high-altitude (2,900 to 3,600 feet) subspecies *V. latastei latastei*. *V. l. gaditana* is like other *Vipera* in having a FX activating procoagulant venom, while *V. latastei latastei* has lost the procoagulant trait (21).

Antivenom neutralization patterns broadly followed the immunizing mixtures. Reflective of having *V. berus* in the immunizing mixture, ViperaTAB was strongly effective only against the *V. berus* venoms. The multi-species immunizing mixture of VIPERFAV (*Vipera ammodytes*, *V. aspis*, *V. berus*) was reflected in its multi-species neutralisation potential. Consistent with Inoserp Europe having a very complex

immunizing mixture (the *Vipera* species *V. ammodytes*, *V. aspis*, *V. berus*, and *V. latastei*, in addition to the *Macrovipera* species *M. lebetina cernovi*, *M. schweizeri*, *M. l. obtusa*, *M. l. turanica* and the *Montivipera* species *M. xanthina*), it displayed the greatest paraspecificity. Consistent with the variation between species and populations within a species observed in this study, previous work using other methods to ascertain variations in other characteristics have similarly reported such variations (43–47). The discrepancy of antivenom neutralizing property on the venom of same species from different locations or within genus is evident in other studies, which is a major issue in antivenom production (42, 48–58).

The comparative testing of the enzyme inhibitors prinomastat and DMPS revealed highly contrasting differences in their specific abilities to neutralize the P-IIIId SVMP responsible for FX activation by the venoms in this study. This is congruent with recent data published for FX activation of neonate *Crotalus culminatus* venom, which prinomastat neutralised but DMPS at the same concentration did not (59) and another report of DMPS requiring long incubation times to exert a discernable effect (60).

Additional important caveats are that this study only examined the neutralization capacity of antivenoms and enzyme-inhibitors using *in vitro* systems. Neutralization assays conducted in this study were under preincubation conditions and as such, demonstrated failure under such idealized conditions are strongly suggestive of failure under dynamic physiological conditions. Thus the limited taxonomical range of ViperaTAB antivenom in this study is strongly suggestive of limited taxonomical range when tested *in vivo* and real-world use, as is the failure of DMPS suggestive of *in vivo* and real-world failure. However, success under such *in vitro* conditions does not automatically translate into success in *in vivo* models or real-world clinical cases. Therefore additional studies must be undertaken using *in vivo* assays and then clinical studies before clinical recommendations can be made. Thus while the Inoserp and VIPERFAV antivenoms, and the enzyme-inhibitor prinomastat, are predicted by this study to have broad utility, this must be corroborated by *in vivo* and subsequent clinical studies.

Another important caveat is that this study focused solely upon the coagulotoxic effects of these venoms. Each of these snake venoms has clinically relevant profile extending beyond coagulopathy, such as neurotoxicity, another major lethal trait (61). In addition to P-IIIId SVMP, these venoms contain myriad other toxins, including snake venom serine protease (SVSP), phospholipase A₂ (PLA₂), and L-Amino acid oxidase (LAAO) (62). In a study on *V. berus* venom, the complex presence of basic phospholipases, SVSPs, LAAO, SVMP, were responsible for hemotoxicity, myotoxicity, cytotoxicity and neurotoxicity (63). *V. aspis* shared similar toxins with *V. berus* along with PLA₂ (ammodytoxin B-like PLA₂; neurotoxic effect), SVMP inhibitor, SVMP, SVSP, and disintegrins; however, a higher presence of disintegrins were seen in *V. aspis* compared to *V. berus* (64, 65). In separate studies on *V. kaznakovi* and *V. anatolica* venom, an abundance of SVMP was evident. However, *V. kaznakovi* had a

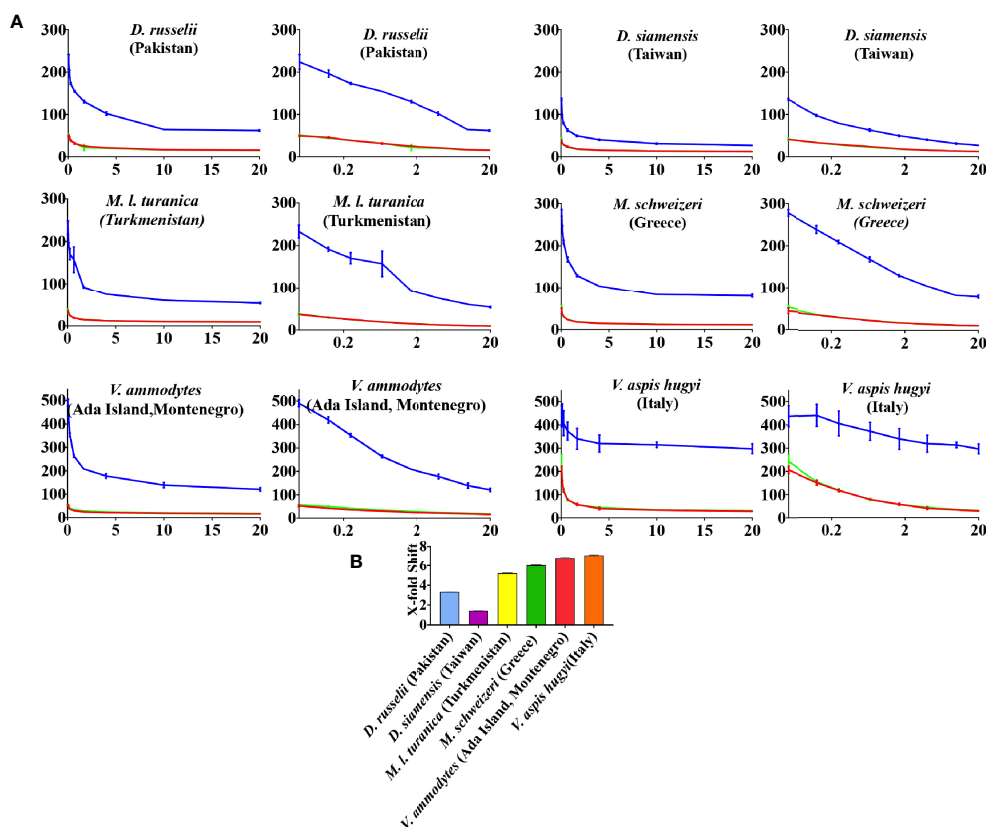


FIGURE 6 | (A) 8-point concentration curves, x-axis showing concentrations of venom in $\mu\text{g}/\text{ml}$ and y-axis showing clotting times in seconds of human plasma with venom and relative inhibitor efficacy. For each species, linear graphs are presented on the left and logarithmic views on the right. Shown are venom-induced clotting times (red curves), effect of venoms after preincubation with prinomastat (final concentration 0.2 mM%; spontaneous control- 484.8 \pm 11.0) (blue curves), and effect of venoms after preincubation with DMPS (final concentration 0.2 mM %; spontaneous control- 425.8 \pm 3.3) (green curves). Values are mean \pm SD of N = 3 and shown as dots with error bars. Some error bars are too small to see and the failure of DMPS to shift the curves results in an identical line to the red (venom only) curves. **(B)** Bar graphs of X-fold magnitude of shift of plasma clotting time due to induction of prinomastat. X-fold magnitude of shift was calculated by the formula $[(\text{AUC of inhibitor + venom}/\text{AUC of venom}) - 1]$. A value of 0 is no shift (no neutralization by inhibitor), while a value above 0 indicates neutralization by inhibitor. Values are mean \pm SD of N = 3.

higher percentage of PLA₂ and SVSP compared to *V. anatolica* (46, 66). *V. nikolskii*, *V. orlovi*, *V. renardii*, and *V. kaznakovi* were all reported to have greater PLA₂ percentage compared to other toxins followed by SVMs (67–69). Great diversity may exist within each of these toxin classes. For example, a SVSP isolated from *V. ammodytes* venom (VaaSP-VX) has been shown to activate Factor X (FX) and Factor V (FV) simultaneously, a function congruent with the metalloproteases in this study (although this toxin is in much lower levels in the venom than the metalloprotease), while another *V. ammodytes* SVSP (VaF1 toxin) has α -fibrinogenolytic activity (70, 71). Another *V. ammodytes* toxin, a myotoxic secreted PLA₂ analogue ammodytin L (AtnL) was reported to cause irreversible atrioventricular (AV) blockade (72, 73). *V. ammodytes meridionalis* has been shown to share similar toxins as well as vipoxin (PLA₂, postsynaptic neurotoxin) (74). The presence of additional toxin actions beyond those examined in this study is

reflected in complex envenomation clinical profiles (61). Thus, more comprehensive *in vitro* assays and also *in vivo* studies must be conducted before clinical recommendations can be finalized regarding treatment options for particular species or populations within a species.

This work provides data useful for predicting potential clinical effects and contributing to the evidence-based design of clinical management strategies. As with *Daboia* and *Macrovipera*, an increase in *Vipera* species sizes was correlated with an increase in the FX activation potency, with the largest species (*V. ammodytes*) possessing the most potently procoagulant venom. Consistent with their multi-species immunizing mixture, both Inoserp Europe and VIPERFAV showed broad paraspecificity. In contrast, and consistent with *V. berus* as the sole immunizing venom, ViperaTab strongly specific for *V. berus*. While the small molecule inhibitor prinomastat nullified the effects being tested in representative venoms, DMPS failed to do so at the same molar

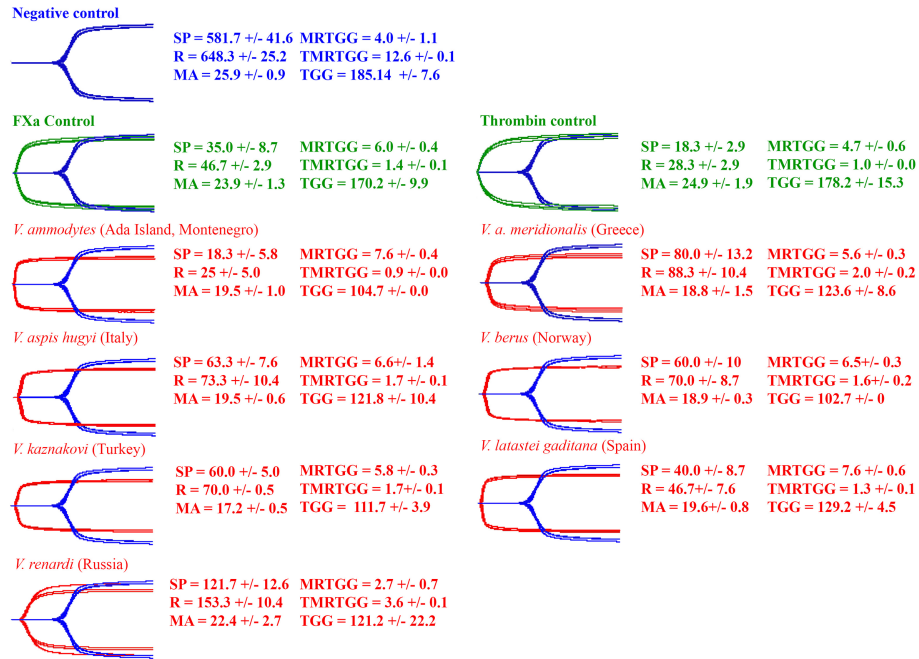


FIGURE 7 | Overlaid thromboelastography traces of spontaneous clotting negative control (blue), Factor X and thrombin positive controls (green) and venom-induced clotting experimental condition (red). Parameters: SP = the split point (time till clot formation begins) (sec); R = time until detectable clot (2 mm +) is formed (sec); MA = maximum amplitude of clot (mm); MRTG = maximum rate of thrombus generation (dynes/cm2/s); TMRTG = time to maximum rate of thrombus generation (min); and TGG = total thrombus generated (dynes/cm2). Values are mean ± SD of N = 3.

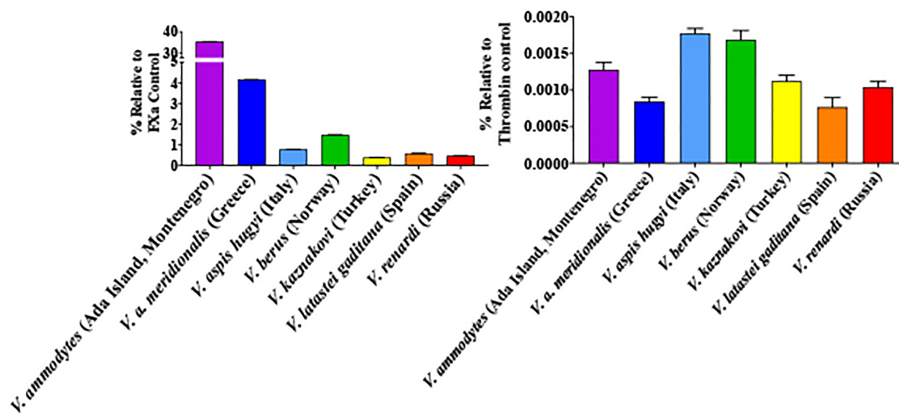
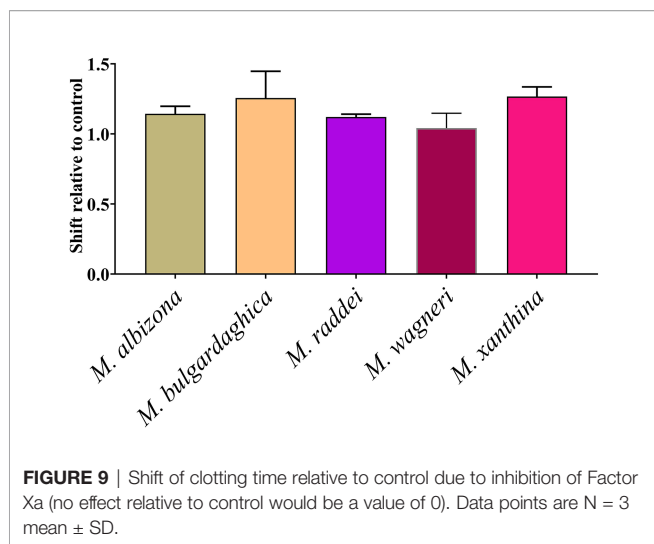


FIGURE 8 | Ability of venoms to activate Factor X (left) compared to prothrombin (right). Results are presented relative to the control of the same amount of the corresponding activated enzyme form (note difference in y-axis scales between the two graph sets). Data points are N = 3 mean ± SD.

concentrations and experimental conditions, including incubation times. In contrast to the retention of the FX activating procoagulant trait in *Vipera* species, *Montivipera* venoms were shown to be unique for the clade, exhibiting anticoagulant activity through the inhibition of FXa, paralleling its specialization for a unique montane habitat. This trend has also been noted for the lowland *V. l. gaditana*, which retained the FX activating procoagulant trait, while the derived high-altitude subspecies *V.*

l. latastei had a secondary reduction in FX potency. This study therefore underscores the importance of studying evolution in parallel to venom biochemistry in order to provide data essential for understanding potential clinical effects of particular species or populations, and the relative therapeutic options.

Future work should examine for prey specific effects to reconstruct the evolutionary shaping pressures by testing several hypotheses emerging from this work regarding the



selection pressures exerted by prey type, prey retaliation potential, and prey escape potential, all of which have been shown to be major drivers of venom evolution (75–77). First is the hypothesis that as mammals are particularly sensitive to procoagulant toxins due to the high circulatory rates making them rapidly subjugated by stroke as a consequence of the large blood clots formed by the venoms, the evolution larger body size is linked to an increased proportion of mammalian prey in the diet (78). This is in turn linked to a second testable hypothesis, that the greater proportion of mammals in the diet, leads to an increased chance of prey retaliation and thus a selection pressure for the increased levels of stroke-inducing procoagulant toxins in order to rapidly subjugate such dangerous prey, as has been seen for mammal specialists such as Australian elapids in the *Oxyuranus* and *Pseudonaja* genera (37). A third testable hypothesis is that the specialization into a high-altitude shift is linked to either a shift in prey type towards amphibian or reptilian prey, thus providing the selection pressure for the down-regulation of the procoagulant venom phenotype and, in the case of *Montivipera* the evolution of the anticoagulant phenotype. A linked fourth testable hypothesis is that the relative prey escape potential is another significant variable driving venom evolution, whereby montane habitats occupied by *Montivipera* and *V. l. latastei* with abundant rock cracks result in higher chance of prey escaping into inaccessible areas, versus snakes living in lowland arid habitats which are able to scent track prey over considerable distances. Such variation in relationship to altitude and prey type and prey escape potential has been noted for the rattlesnake species *Crotalus helleri* (79). In addition, as all venom samples used in this study from adult specimens, future work should examine ontogenetic shifts to

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ascertain if juvenile snakes have differentially procoagulant venoms, which has been noted for other species (59, 78, 80, 81).

This work thus has a broad impact, contributing to the understanding of the lethal coagulopathy produced by some species, while also providing a starting point for diverse evolutionary studies. We hope these findings stimulate further research into the evolution of venom in this group of fascinating snakes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UQ Human Ethics Committee Approval #2016000256 using pooled plasma from anonymous patients, supplied by the Australian Red Cross under Research Agreement #18-03QLD-0. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by UQ NEWMA approval # 2021/AE000075.

AUTHOR CONTRIBUTIONS

Study conception: BF. Study design AC, CZ, ML, RC, MA, and RS. Resources ML, RC, TJ, HH, MA, and RS. Conducting of experiments. AC, CZ, and BF. Data analysis. AC, CZ, and BF. Primary draft writing AC and BF. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: ML was employed by the company Ophirex, MA by Micropharm, and RS by Inosan Biopharma, all of which made products tested in this manuscript. However, the companies had no input in experimental design or reviewing of results before publication.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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