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Proteomic profiling of dog urine after European adder (*Vipera berus berus*) envenomation by two-dimensional difference gel electrophoresis

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ABSTRACT

Between April and September every year, many dogs in Finland are bitten by *Vipera berus berus*, also known as the European adder, the only venomous snake in the area. Exposure to snake bite venom causes local and systemic symptoms and in severe cases can lead to death. Urine samples were collected from four dogs bitten by *V. berus berus* and treated in the intensive care unit of the Veterinary Teaching Hospital at the University of Helsinki. The inclusion criteria were a strong suspicion of an adder bite no more than two days before admission and clinical signs of an adder bite. Exclusion criteria were defined as ongoing treatment with glucocorticoids or a known history of liver or kidney diseases. Six privately owned, healthy dogs were obtained as controls. Samples were subjected to 2D-DIGE analysis. Image analysis was performed with DeCyder 7.0 2D software, and protein spots demonstrating a minimum 1.5-fold difference in average spot volume ratios between envenomed and control dogs with a Student's *t*-test *p*-value of less than 0.05 were picked and identified using LC–MS/MS. In 2D-DIGE analysis, seven proteins were significantly ($p < 0.05$) over-expressed in the urine of dogs bitten by *V. berus berus* compared to the control group. From these, five proteins were identified: beta-2-microglobulin (b2MG), alpha-1-antitrypsin (AAT), albumin, fetuin-B and superoxide dismutase (SOD1). Results indicate that envenomation by *V. berus berus* alter the urinary protein profile in dogs.

1. Introduction

Every year, many dogs in Scandinavia are bitten by *Vipera berus berus*, also known as the European adder, mainly in the period from April to September. The European adder is geographically widely distributed throughout Europe and Asia (Weinelt et al., 2002; Karlson-Stieber et al., 2006), and is the only venomous snake in Scandinavia. The snake venom consists of a mixture of proteins with toxic and enzymatic properties, such as proteases, peptide hydrolases, hyaluronidases, phospholipases, metalloproteinases, phosphodiesterase, and L-amino acid oxidase (Calderon et al., 1993; Reading, 1996; Samel et al., 2006; Terra et al., 2009). The exact composition of *V. berus berus* venom is unknown, but venoms of *Vipera* species share characteristics at the familial and generic level, although variations in venom composition due to seasonal, individual, and geographical differences have been reported (Master and Kornalik, 1995; Gubensek et al., 1974; Jayanthi and Gowda, 1988; Tan and Ponnudurai, 1990; Georgieva et al., 2008). In rabbits, viper (*Vipera aspis*) venom has a terminal circulatory half-life of 12 h, suggesting that most of the venom is eliminated within three days of envenomation (Auderbert et al., 1993). Only a small percentage of the venom was found to be excreted in urine (Auderbert et al., 1993). *In vitro* studies have demonstrated that snake venom of different

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species can be detected in kidney tissue after envenomation, and in some cases has a direct nephrotoxic effect (Ratcliffe et al., 1989; Burdmann et al., 1993; de Castro et al., 2004). This has now proven to be true also in *in vivo* studies (Mandal and Bhattacharyya, 2007; De Roodt et al., 2012).

Approximately 70% of the reported *V. berus berus* bites cause no or very mild effects in humans, and deaths rarely occur (Reading, 1996). Bites without envenomation have been observed in approximately 10% of cases (Petite, 2005; Karlson-Stieber et al., 2006). Local symptoms such as swelling, bruising and pain in the area of the bite are the most prominent manifestation in dogs and humans (Reading, 1996; Grönlund et al., 2003; Karlson-Stieber et al., 2006; Lervik et al., 2010). Systemic symptoms such as shock, cardiovascular and gastrointestinal symptoms, CNS depression, acute kidney injury and respiratory distress occur in more severe cases in humans, dogs, and horses (Grönlund et al., 2003; Karlson-Stieber et al., 2006; Lervik et al., 2010; Anlén, 2011).

The primary aim of this study was to detect urinary proteins by two-dimensional difference gel electrophoresis from dogs bitten by *V. berus berus* and to compare them with urinary proteins from healthy controls. The secondary aim was to investigate whether envenomation causes acute kidney injury in dogs.

2. Materials and methods

2.1. Animals

Eleven dogs bitten by *V. berus berus* and treated in the intensive care unit at the Veterinary Teaching Hospital of University of Helsinki were obtained for this study. The inclusion criteria were a strong suspicion of a viper bite (owner saw the dog being bitten or saw a viper close to the dog) no more than two days before admission, clinical signs of a viper bite, and an increased urinary protein-to-creatinine ratio. Clinical signs of the bite were defined as swelling in the bite area, severe pain, distress, discomfort and hypovolemia. Ongoing treatments with glucocorticoids or a known history of liver or kidney diseases were defined as exclusion criteria. Treatment in the intensive care unit comprised intravenous fluid therapy. Opioid analgesics, antibiotics, gastroprotective medications and antiemetics were given when needed. Four dogs were treated with antivenom. Eight privately owned, healthy dogs were obtained as controls. More detailed information on the dogs used in 2D-DIGE and verification analysis in this study can be found in the Supplementary data.

2.2. Sample collection

Single void urine samples were collected in the intensive care unit from dogs bitten by a European adder. The owners of the healthy control dogs provided a urine sample from the morning void. The urinary creatinine and protein concentrations were measured using routine laboratory methods, and urine was centrifuged (1300 *g*, 10 min) at 4 °C to remove cellular components and debris, and frozen at −80 °C until analyzed.

2.3. 2De-DIGE analysis

2.3.1. Sample preparation and CyDye labeling

The urine from four dogs bitten by a European adder and six control dogs was chosen for 2D-DIGE analysis. The criterion for selection was the volume of urine obtained. None of the dogs had received antivenom treatment. Urine samples were first concentrated and desalted by centrifugal ultrafiltration using Amicon Ultra-0.5 10 K centrifugal units (Millipore). The samples were then precipitated with trichloroacetic acid–acetone precipitation and solubilized in 50 μl of labeling buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris). The concentrations of proteins in the solubilized samples were measured using a 2D Quant Kit (GE Healthcare) according to the manufacturer's instructions. Proteins from the control group were pooled due to the low protein concentration. The samples were then labeled with Cy2, Cy3 and Cy5 dyes (CyDye DIGE Fluor minimal dyes, GE Healthcare) according to the Ettan two-dimensional difference gel electrophoresis (DIGE) protocol. Briefly, 50 μg of protein from each sample was labeled with 400 pmol of the Cy3 and Cy5 dyes. Internal standard labeled with Cy2 dye was established by combining 25 μg of each sample. The labeling reaction was incubated for 30 min on ice in the dark and halted by adding 1 mM lysine to the reaction following 10 min of incubation as earlier. The labeled samples were pooled and separated by two-dimensional gel electrophoresis as detailed below.

2.3.2. Two-dimensional gel electrophoresis

The labeled proteins were analyzed by two-dimensional difference gel electrophoresis as described earlier (Ünlü et al., 1997). An immobilized pH gradient (IPG) strip (24 cm, pH 3–10, nonlinear, GE Healthcare) was used for isoelectric focusing. IPG strips were loaded with 150 μg of protein in total by using a cup-loading method. Isoelectric focusing was performed using IPGPhor (GE Healthcare) at 20 °C as follows: 3 h at 150 V, 3 h at 300 V, then linear ramping to 10 000 V, and 10 000 V for 50 000 V, with the maximum current per strip being 75 μA. After focusing, the isoelectric strips were prepared for the second dimension gels by incubation for 15 min in equilibrium buffer solution I (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 0.2% Bromophenol blue, with added 10 mg/ml dithiothreitol (DTT)). This was followed by equilibration for another 15 min in buffer solution II (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.2% Bromophenol blue, supplemented with 25 mg/ml iodoacetamide). The prepared IPG strips were then placed on 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and sealed with overlay agarose (Bio-Rad). Electrophoresis was initiated at 50 V for 30 min, which was followed by 400 V for 3 h. The gels were scanned between low-fluorescence glass plates using an FLA-5100 laser scanner (Fujifilm) at wavelengths of 473 (for Cy2), 532 (for Cy3), and 635 nm (Cy5) using voltages of 420, 410, and 400 V. After scanning, the gels were silver stained as described previously (O'Connel and Stults, 1997).

2.3.3. Image analysis and data processing

The gel images were analyzed and statistically assessed using DeCyder 7.0 software (GE Healthcare). First, the gels

Table 1
Proteins detected by LC–MS/MS expressed differently in the urine of dogs bitten by *Vipera berus berus* compared to controls.

Spot	Identified protein	Accession number	Theoretical pI/MW (Da)	Matched peptides	Sequence coverage %	Mascot score	Ratio	*P*-value
2	Alpha-1-antitrypsin	giÐ 121583756	5.58/46.3	24	16.2	558	3.05	0.0042
4	Albumin	giÐ 3319897	5.52/68.6	36	41.6	1043	2.73	0.0036
9	Beta-2-microglobulin	XP_535458	5.92/14.2	22	28.6	307	8.42	0.0061
11	Fetuin-B	giÐ74003556	5.64/42.3	8	37.1	478	4.4	0.0088
12	Superoxide dismutase (Cu–Zn)	Q8WNN6	5.69/15.6	9	21.6	235	2.66	0.0091

pI: isoelectric point, MW: molecular weight.

were automatically analyzed using the batch processor function to normalize the Cy2, Cy3 and Cy5 images from each gel. Spot volumes were calculated and compared with Cy2 volumes (internal standard) to correct the inter-gel variations. In the biological variation module the Cy2 images of all eight gels were matched and the spot volumes were compared. Approximately 1400 separate spots were detected on each gel. Protein spots demonstrating a minimum 1.5-fold difference in average spot volume ratios between groups in all biological replicates and having a Student's *t*-test *p*-value of less than 0.05 were picked and identified.

2.3.4. Protein identification

Protein spots of interest were manually excised from the gel and digested in-gel using trypsin (Trypsin Gold, mass spectrometry grade, Promega) as described earlier (Schevchenko, 1996; Jensen et al., 1998). The samples were first concentrated and desalted on a C_{18} trap column (PROTECOL, SGE Analytical Science, Griesham, Germany) followed by peptide separation on a PepMap 100 C_{18} analytical column (LC Packings, Sunnyvale, CA). MS/MS of peptides was performed on a hybrid quadrupole/TOF mass spectrometer with a Nanospray II source (QSTAR Elite, applied Biosystems, Foster City, CA). Proteins were identified using the local Mascot version 2.2 (Matrix Science, London, UK) against the in-house database. The search criteria included digestion with one missed cleavage allowed, carbamidomethyl modification of cysteine as a fixed modification, and oxidation of methionine as a variable modification.

2.4. Verification of identified proteins

The identified proteins were biologically validated by ELISA or Western blotting. The Western blot analysis of urinary AAT, Fetuin-B and SOD1 was performed on the urine of three dogs bitten by a European adder included in 2D-DIGE analysis. ELISA analysis of b2MG was performed on the urine of nine bitten dogs and four healthy dogs according to the manufacturer's instructions (Cusabio Biotech Co., LTD).

3. Results

3.1. Urinary creatinine and protein concentrations

The urinary creatinine concentration (mean ± standard deviation [min–max]) was 179.6 ± 106.2 mg/dL (92.1–439.4 mg/dL) in healthy control dogs and 37.2 ± 32.8 mg/dL (6.7–123.4 mg/dL) in dogs bitten by *V. berus berus*. The total urinary protein concentration (mean ± standard deviation [min–max]) was 15.0 ± 6 mg/dL (6.3–27.2 mg/dL) in healthy controls and 39.78 ± 34.31 mg/dL (3.2–125.1 mg/dL) in dogs bitten by adder. The respective urinary total protein-to-creatinine ratios were 0.09 ± 0.03 mg/mg (0.05–0.13 mg/mg) and 1.97 ± 1.99 mg/mg (0.07–5.82 mg/mg).

3.2. 2D-DIGE findings

In 2D-DIGE analysis, eleven proteins were significantly differentially expressed in the urine of dogs bitten by *V. berus berus* compared to the control group. Seven urinary proteins were upregulated after envenomation by *V. berus berus*, and five of them were identified by LC–MS/MS: beta-2-microglobulin (b2MG), alpha-1-antitrypsin (AAT), albumin, Fetuin-B and superoxide dismutase (SOD1) (Table 1) (Fig. 1, Fig. 2). Four proteins were downregulated and were not chosen for identification by mass spectrometry. All identified proteins were in the expected size and pI ranges in 2D analysis.

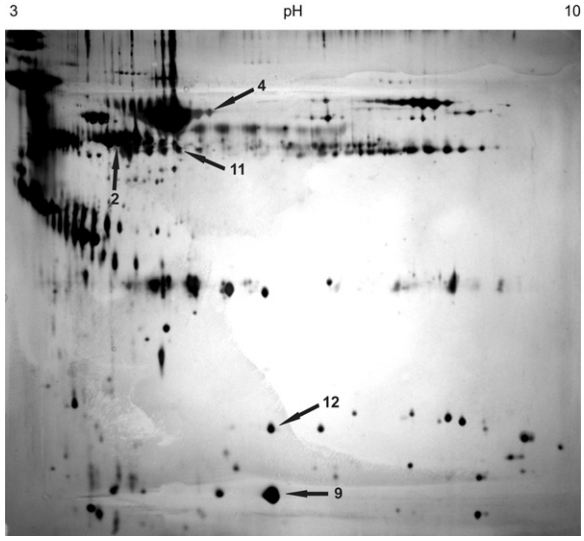

Fig. 1. A representative image of a 2D-DIGE gel after silver staining showing protein spots successfully identified with LC–MS/MS that were significantly differently expressed between dogs bitten by *Vipera berus berus* and healthy dogs. Protein spots are marked with arrows and numbered: 2. Alpha-1-antitrypsin, 4. Albumin, 9. Beta-2-microglobulin, 11. Fetuin-B, 12. Superoxide dismutase 1.

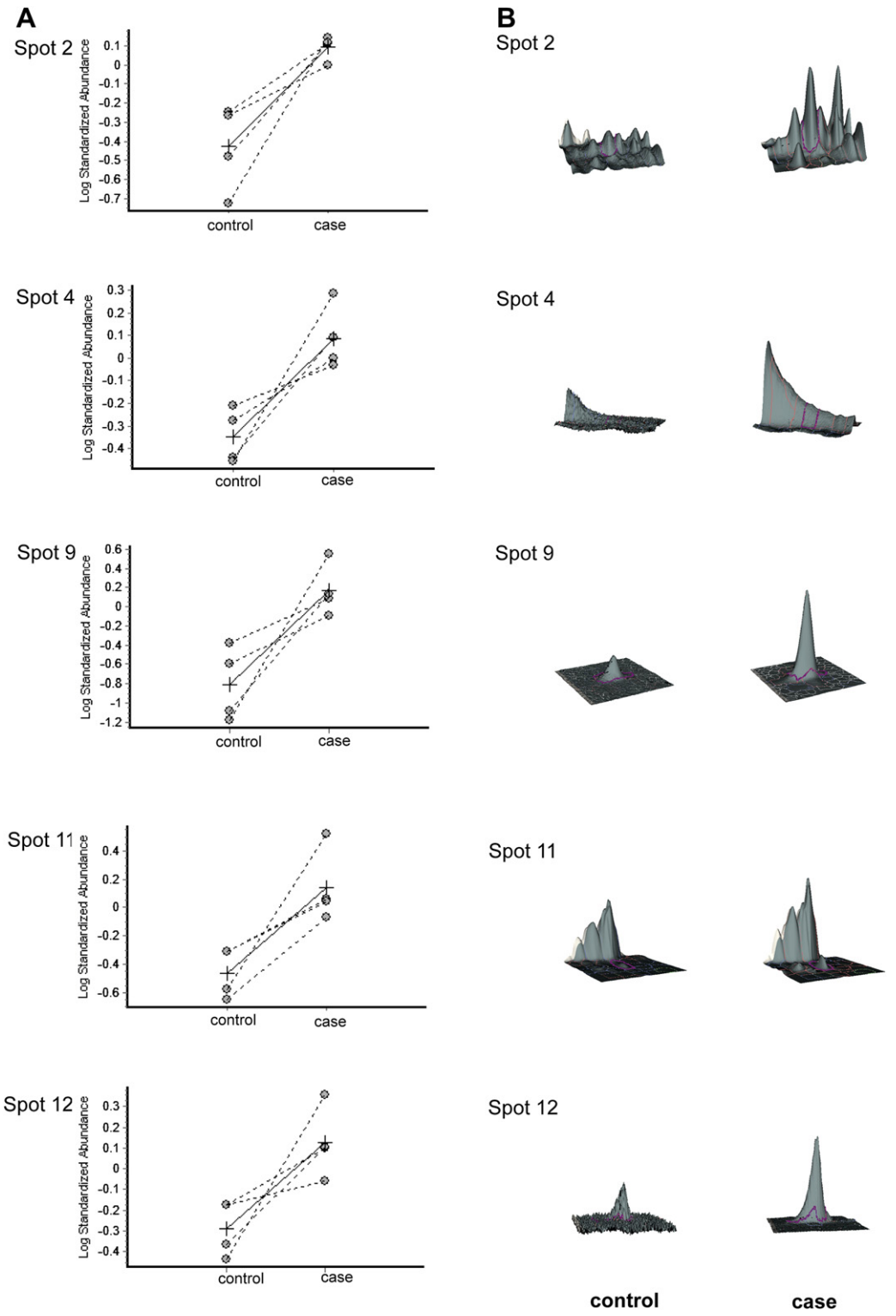

Fig. 2. Graphical representation of the standardized log abundance (i.e., log abundance of Cy3- or Cy5-labeled spot over log abundance of Cy2-labeled standard spot). Individual lines show each of the four biological replicates from cases and controls. The three-dimensional fluorescence intensity profiles of the individual spots shown for one of the biological replicates comparing case and control of each of the five identified protein spots that showed significant changes. Proteins are: spot 2. Alpha-1-antitrypsin, spot 4. Albumin, spot 9. Beta-2-microglobulin, spot 11. Fetuin-B, spot 12. Superoxide dismutase 1.

3.3. Verification of identified proteins

The identified proteins were verified by Western blotting (AAT, Fetuin-B and SOD1) (Fig. 3) or ELISA (b2MG). Western blot results verified the identification of the proteins. In ELISA analysis the results were expressed as ratios to the urine creatinine concentration (b2MG creatinine index). The b2MG creatinine index (mean ± standard deviation [min–max]) of nine dogs bitten by *V. berus berus* was 0.25 ± 0.22 μg/mg (0.04–0.78 μg/mg), while in the control dogs the index was 0.050 ± 0.007 μg/mg (0.04–0.05 μg/mg).

4. Discussion

Urinary excretion of serum derived proteins such as albumin, AAT and b2MG is considered to provide markers for tubular injury (Yanagisawa et al., 1983; Herget-Rosenthal et al., 2004). Albumin, the most abundant plasma protein, is produced in the liver and excreted to the bloodstream. Albumin is filtered in the glomeruli and reabsorbed in the proximal tubuli, although the kidneys also appear to produce albumin locally in response to acute kidney injury (Ware et al., 2011). Albuminuria has commonly been considered as a marker of renal disease. The increased urine protein concentration detected in the affected dogs was probably caused by increased glomerular permeability and decreased reabsorption of proteins due to tubular injury. Thus also fluid reabsorption may have been unbalanced resulting polyuria with diluted urine. Treatment of the affected dogs contained substantial intravenous fluid therapy which had probably also diluted urine and therefore decreased urine creatinine concentration.

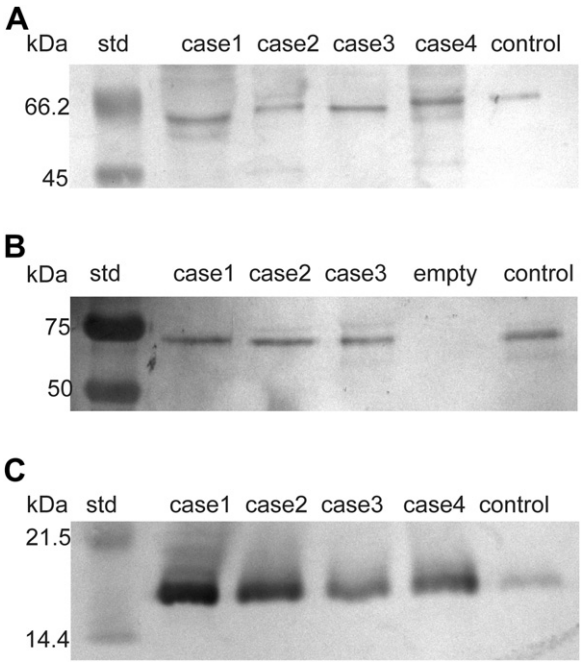

Fig. 3. Western blot analysis of urine samples from three dogs bitten by *Vipera berus berus* (10 μg of total protein of cases, 20 μg of total protein of control). The proteins were separated on 12% SDS-PAGE and they were detected using polyclonal anti-alpha-1-antitrypsin (A), polyclonal anti-fetuin-B (B) and polyclonal anti-superoxide dismutase 1 (C).

Alpha-1-antitrypsin (AAT) is an acute-phase glycoprotein produced in the liver, from where it is secreted into the bloodstream. It belongs to the serine protease inhibitor (SERPIN) family, acts as an inhibitor of many proteases, and possesses serine-type endopeptidase inhibitor activity (Gettins, 2002). To protect the extracellular matrix during inflammation, AAT inhibits elastase secreted by neutrophils, and contributes to the regulation of proteolysis (du Bois et al., 1991). AAT was shown to contribute to kidney protection by anti-apoptotic and anti-inflammatory routes in renal ischemic/reperfusion injury (Daemen et al., 2000). It is considered to be one of the biomarkers for AKI in humans (Metzger et al., 2010).

Beta-2-microglobulin (b2MG) is a part of the major histocompatibility complex (MHC) class I molecules and is filtered by the renal glomerulus (Schardijn and van Eps, 1987). b2MG is reabsorbed from epithelial cells of the proximal tubule by megalin- and cubilin-mediated endocytosis and degraded to amino acids (Schardijn and van Eps, 1987; Verroust et al., 2002). Increased excretion of b2MG as well as other low-molecular-weight proteins indicates acute kidney injury well before serum creatinine levels increase (Metzger et al., 2010). An increased serum b2MG concentration has also been shown to indicate reduced renal function more accurately than increased serum creatinine (Bianchi et al., 2001). In our study, the b2MG creatinine index of control dogs was in line with earlier reports (Vittinghus, 1990; Nakajima et al., 2001). The increased urinary excretion of albumin, AAT and b2MG in this study suggests that envenomation by *V. berus berus* causes tubular dysfunction in dogs.

Fetuin-B is an extracellular protein that functions in cysteine-type endopeptidase inhibitor activation. It belongs to the cystatin superfamily of proteases and is a paralogue to fetuin-A, sharing some of the functions (Olivier et al., 2000; Denecke et al., 2003). Both fetuin-A and fetuin-B are mainly expressed in the liver in adult mice, from where they are secreted to the bloodstream (Denecke et al., 2003). The expression of both fetuins was also detected in the kidneys and tongue, and fetuin-B alone in the lungs and ovaries, all organs that possess secreting epithelia (Denecke et al., 2003). Fetuin-A has been detected in urinary exosomes after acute kidney injury in rats and humans, and is considered to be a biomarker for AKI (Zhou et al., 2006; Devarajan, 2008). In this study the exosomes were not isolated, suggesting that the source of excreted fetuin-B is not the exosomes but rather the plasma.

Copper/zinc superoxide dismutase (SOD1) is a cytosolic enzyme belonging to a metal-containing enzyme family that catalyzes the dismutation of superoxide anions and is expressed in all mammalian tissues, especially the liver and kidneys (Marklund et al., 1982; Takada et al., 1982; Frederiks and Bosch, 1997). These enzymes act as the most important line of antioxidant defense against reactive oxygen species (ROS), metabolic by-products that can lead to oxidative stress (Zelko et al., 2002; Johanson and Giulivi, 2005). Oxidative stress has been associated with kidney

injury induced by *Crotalus durissus terrificus* envenomation in mice (Yamasaki et al., 2008). SOD1 has been identified as a urinary marker for carbon tetrachloride-induced hepatotoxicity in rats (Smyth et al., 2008). The excretion of SOD1 in this study suggests that oxidative stress is also associated with *V. berus berus* envenomation in dogs.

In conclusion, envenomation by *V. berus berus* causes an altered urinary proteomic profile in dogs. The excreted alpha-1-antitrypsin (AAT), beta-2-microglobulin (b2MG) and albumin are well-studied proteins associated with tubular injury. More studies are needed to understand the role and origin of fetuin-B and copper/zinc superoxide dismutase (SOD1) in dog urine after a European adder bite, and their sensitivity and specificity as potential biomarkers.

Ethical statement

The authors guarantee that the works presented in this manuscript follow the rules of ethics and respect the duties of authors presented in the Elsevier's Ethical Guidelines for Journal Publication.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2012.08.010.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

References

Anlén, K.G., 2011. Effects of bites by the European adder (*Vipera berus*) in seven Swedish horses. Vet. Rec. 162, 652–656.

Auderbert, F., Urtizberea, M., Sabouraud, A., Scherrmann, J.-M., Bon, C., 1993. Pharmacokinetics of *Vipera aspis* venom after experimental envenomation in rabbits. J. Pharmacol. Exp. Ther. 268, 1512–1517.

Bianchi, C., Donadio, C., Tramonti, G., Consani, C., Lorusso, P., Rossi, G., 2001. Reappraisal of serum β2-microglobulin as marker of GFR. Ren. Fail. 23, 419–429.

Burdmann, E.A., Woronik, V., Prado, E.B.A., Abdulkader, R.C., Saldanha, L.B., Barretto, O.C.O., Marcondes, M., 1993. Snakebite-induced acute kidney failure: an experimental model. Am. J. Trop. Med. Hyg. 48, 82–88.

Calderon, L., Lomonte, B., Gutierrez, J.M.,, Tarkowski, A., Hanson, L.A., 1993. Biological and biochemical activities of *Vipera berus* (European viper) venom. Toxicon 31 (6), 743–753.

Daemen, M.A., Heemskerk, V.H., van't Veer, C., Denecker, G., Wofs, T.G., Vandenabeele, P., Buurman, W.A., 2000. Functional protection by acute phase proteins α_1-acid glycoprotein and α_1-antitrypsin against ischemia/reperfusion injury by preventing apoptosis and inflammation. Circulation 102, 1420–1426.

de Castro, I., Burdmann, E., de, A., Seguro, A.C., Yu, L., 2004. *Bothrops* venom induces direct renal tubular injury: role for lipid peroxidation and prevention by antivenom. Toxicon 43, 833–839.

De Roodt, A.R., Lago, N.R., Stock, R.P., 2012. Myotoxicity and nephrotoxicity by *Micrurus* venoms in experimental envenomation. Toxicon 59, 356–364.

Denecke, B., Gräber, S., Schäfer, C., Heiss, A., Wöltle, M., Jahnen-Dechent, W., 2003. Tissue distribution and activity testing suggest a similar but not identical function of Fetuin-B and fetuin-A. Biochem. J. 376, 135–145.

Devarajan, P., 2008. Proteomics for the investigation of acute kidney injury. In: Proteomics in Nephrology – Towards Clinical Applications. Contrib. Nephrol., vol. 160, pp. 10–25.

du Bois, R.M., Bernaudin, J.F., Paakko, P., Hubbard, R., Takahashi, H., Ferrans, V., Crystal, R.G., 1991. Human neutrophils express the alpha 1-antitrypsin gene and produce alpha 1-antitrypsin. Blood 77, 2724–2730.

Frederiks, W.M., Bosch, K.S., 1997. Localization of superoxide dismutase activity in rat tissues. Free Rad. Bio Med. 22, 241–248.

Georgieva, D., Risch, M., Kardas, A., Buck, F., von Bergen, M., Betzel, C., 2008. Comparative analusis of the venom proteomes of *Vipera ammondytes ammondytes* and *Vipera ammondytes meridionalis*. J. Proteome Res. 7, 866–886.

Gettins, P.G.W., 2002. Serpin structure, mechanism and function. Chem. Rev. 102, 4751–4803.

Grönlund, J., Vuori, A., Nieminen, S., 2003. Adder bites. A report of 68 cases. Scand. J. Surg. 92, 171–174.

Gubensek, F., Sket, D., Turk, V., Lebez, D., 1974. Fractionation of *Vipera ammondytes* venom and seasonal variation of its composition. Toxicon 12 (2), 167–168.

Herget-Rosenthal, S., Poppen, D., Hüsing, J., Marggraf, G., Pietruck, F., Jakob, H.-G., Philipp, T., Kribben, A., 2004. Prognostic value of tubular proteinuria and enzymuria in nonoligouric acute tubular necrosis. Clin. Chem. 50 (3), 552–558.

Jayanthi, G.P., Gowda, T.V., 1988. Geographical variation in India in the composition and lethal potency of Russel's viper (*Vipera russeli*) venom. Toxicon 26 (3), 257–264.

Jensen, O.N., Larsen, M.R., Roepstorff, P., 1998. Mass spectrometric identification and microcharacterization of proteins from electrophoretic gels: strategies and applications. Proteins, 74–89.

Johanson, F., Giulivi, C., 2005. Superoxide dismutases and their impact upon human health. Mol. Aspects Med. 26, 340–352.

Karlson-Stieber, C., Salmonson, H., Persson, H., 2006. A nationwide study of *Vipera berus* bites during one year – epidemiology and morbidity of 231 cases. Clin. Toxicol. 44, 24–30.

Lervik, J.B., Lilliehöök, I., Frendin, J.H.M., 2010. Clinical and biochemical changes in 53 Swedish dogs bitten by the European adder – *Vipera berus*. Acta Vet. Scand. 52, 26–37.

Mandal, S., Bhattacharyya, D., 2007. Ability of a small, basic protein isolated from Russell's viper venom (*Daboia russeli russeli*) to induce renal tubular necrosis in mice. Toxicon 50, 236–250.

Marklund, S.L., Westman, G., lundgren, E., Roos, G., 1982. Copper- and zinc-containing superoxide dismutase, mangane-containing superoxide dismutase, catalase and glutathione peroxidase in normal and neoplastic human cell lines and normal human tissues. Cancer Res. 42, 1955–1961.

Master, R.W., Kornalik, F., 1995. Biochemical differences in yellow and white venoms of *Vipera ammodytes* and Russell's viper. J. Biol. Chem. 240 (1), 139–142.

Metzger, J., Kirch, T., Schiffer, E., Ulger, P., Mentes, E., Brand, K., Weissinger, E.M., Haubitz, M., Mischack, H., Herget-Rosenthal, S., 2010. Urinary excretion of twenty peptides forms an early and accurate diagnostic pattern of acute kidney injury. Kidney Int. 78, 1252–1262.

Nakajima, Y., Hoshi, F., Higuchi, S., Kawamura, S., 2001. Determination of canine β_2-microglobulin in plasma and urine by enzyme-linked immunosorbent assay. J. Vet. Med. Sci. 63 (3), 343–345.

Olivier, E., Soyry, E., Ruminy, P., Husson, A., Parmentier, F., Daveau, M., Salier, J.-P., 2000. Fetuin-B, a second member of the fetuin family in mammals. Biochem. J. 350, 0589–0597.

O'Connel, K.L., Stults, J., 1997. Identification of mouse liver proteins on two-dimensional electrophoresis gels by matrix-assisted laser desorption/ionization mass spectrometry of *in situ* enzymatic digests. Electrophoresis 18, 349–359.

Petite, J., 2005. Viper bites: treat or ignore? Swiss Med. Wkly. 135, 618–625.

Ratcliffe, P.J., Pukrittayakamee, S., Ledingham, J.G.G., Warrell, D.A., 1989. Direct nephrotoxicity of Russell's viper venom demonstrated in the isolated perfused rat kidney. Am. J. Trop. Med. Hyg. 40, 312–319.

Reading, C.J., 1996. Incidence, pathology, and treatment of adder (*Vipera berus L*) bites in man. J. Accid. Emerg. Med. 13, 346–351.

Samel, M., Vija, H., Rönnholm, G., Siigur, J., Kalkkinen, N., Siigur, E., 2006. Isolation and characterization of an apoptotic and platelet aggregation inhibiting L-amino acid oxidase from *Vipera berus berus* (common viper) venom. Biochim. Biophys. Acta 1764, 707–714.

Schardijn, G.H.C., van Eps, S.L.W., 1987. Beta-2-microglobulin: its significance in the evaluation of renal function. Kidney Int. 32, 635–641.

Schevchenko, A., 1996. Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. Anal. Chem. 68 (5), 850–858.

Smyth, R., Turton, J.A., Clarce, C.J., York, M.J., Dare, T.O., Lane, C.S., Munday, M.R., 2008. Identification of superoxide dismutase as a potential urinary marker of carbon tetrachloride-induced hepatic toxicity. Food Chem. Toxicol. 46, 2972–2983.

Takada, Y., Noguchi, T., Okabe, T., Kajiyama, M., 1982. Superoxide dismutase in various tissues from rabbits bearing the Vx-2 carcinoma in the maxillary sinus. Cancer Res. 42, 4233–4235.

Tan, N.-H., Ponnudurai, G., 1990. A comparative study of the biological properties of venoms from snakes of the genus *Vipera* (true adders). Comp. Biochem. Physiol. 96b (4), 683–688.

Terra, R.M.S., Pinto, A.F.M., Guimaraes, J.A., Fox, J.W., 2009. Proteomic profiling of snake venom metalloproteinases (SVMPs): insights into venom induced pathology. Toxicon 54, 836–844.

Ünlü, M., Morgan, M.E., Minden, J.S., 1997. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18, 2071–2077.

Verroust, P.J., Birn, H., Nielsen, R., Kozyraki, R., Christensen, E.I., 2002. The tandem endocytic receptors megalin and cubulin are important proteins in renal pathology. Kidney Int. 62, 745–756.

Vittinghus, E., 1990. Preanalytical handling of stored urine samples, and measurement of beta 2-microglobulin, orosomucoid, albumin, transferrin and immunoglobulin G in urine by enzyme-linked immunosorbent assays (ELISA). Scand. J. Clin. Lab. Invest. 50, 843–849.

Ware, L.B., Johnson, A.C.M., Zager, R.A., 2011. Renal cortical albumin gene induction and urinary albumin excretion in response to acute kidney injury. Am. J. Physiol. Renal Physiol. 300, F628–F638.

Weinelt, W., Sattler, R.W., Mebs, D., 2002. Persistent paresis of the facial muscle after European adder (*Vipera berus*) bite on the forehead. Toxicon 40, 1627–1629.

Yamasaki, S.C., Villarroel, J.S., Barone, J.M., Zambotti-Villela, L., Silveira, P.F., 2008. Aminopeptidase activities, oxidative stress and renal function in *Crotalus durissus terrificus* envenomation in mice. Toxicon 52, 445–454.

Yanagisawa, H.Y.U., Forbes, M.A., Cooper, E.H., Crockson, R.A., MacLennan, I.C.M., 1983. Aplha-1-microglobulin: an indicator protein for renal tubular function. J. Clin. Pathol. 36, 253–259.

Zelko, I.N., Mariani, T.J., Folz, R.J., 2002. Superoxide dismutase multigene family: a comparison of the CuZn–SOD (SOD1), Mn–SOD (SOD2) and EC–SOD (SOD3) gene structures, evolution and expression. Free Radic. Biol. Med. 33, 337–349.

Zhou, H., Pisitkun, T., Aponte, A., Yuen, P.S.T., Hoffert, J.D., Yasuda, H., Hu, X., Chawla, L., Shen, R.-F., Knepper, M.A., Star, R.A., 2006. Exosomal Fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. Kidney Int. 70, 1847–1857.