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Comparative venom toxin analyses of Nigerian *viperidae* and *elapidae* snakes

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ABSTRACT

Envenoming by snakebite is a serious health problem that maims and kills a large number of people, primarily in rural areas of developing African countries. The first comparative venom proteomic analyses of four snakes from the *viperidae* (*E. ocellatus* and *B. arietans*) and *elapidae* (*N. haje* and *N. katiensis*) families are presented in this study. Two-dimensional electrophoresis was combined with matrix-assisted laser desorption ionization time-of-flight mass spectrometry to analyze the venoms. Proteins were identified by comparing mass spectrometry spectra to those in the reviewed Uniprot-Serpentes database. A protein spot was considered differentially present between samples at a p-value of < 0.05 and a fold change of >2. Viper venoms contained cytotoxic-inducing proteins such as SVMPs, SVSPs, and cytotoxins, whereas elapid snake venoms contained neurotoxic proteins such as PLA_2, 3-FTx, and neurotoxins. The PDQuest annotated protein spots on the 2-DE gels showed that the proteins in these snakes' venoms were differentially expressed between snake families and species. The elapid venoms were predominantly acidic (low pI) with low molecular masses, whereas the viperid venoms had high molecular masses and a pI in the region of 7. Venom phosphodiesterase, L-amino acid oxidase and cysteine-rich venom protein were common in the venoms of these snakes, while an uncommon protein actiflagelin was detected in the *Naja* venoms. Our findings show that there is significant variation in the toxin profiles of these snakes, both at the species and family levels. This has an impact on the clinical manifestations of envenomation. A thorough understanding of the various toxins found in venomous snakes may aid in the development of new and improved therapeutic strategies.

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Background

Despite significant efforts to improve its management, snakebite envenoming remains a public health challenge. Due to the high number of disabilities and deaths it causes, the World Health Organization classified it as a neglected tropical disease [1]. The majority of fatalities occur in tropical and subtropical countries, where rural residents are victims of snakebite [2]. They are vulnerable due to frequent contact with snakes, which occurs primarily during the rainy season, which coincides with the snakes' breeding season [2]. Agricultural workers, including young males and children, are particularly vulnerable [3,4]. Venom infiltration into the human body causes fatal hemorrhage, irreversible kidney failure, tissue damage, permanent disabilities such as amputations, and death [4]. Snakes are found in warmer climates of tropical countries, particularly Africa, Asia, Latin America, and Oceania, due to their ectothermic nature [2,5]. About 20% of snakebite cases occur in Africa [6], with an annual snakebites index of 580 000 [4]. The annual number of snakebites in Nigeria was recently estimated to be 43,000, with 19,000 deaths [3].

The vipers and elapids are the most venomous snakes and are collectively responsible for over 95% of the reported cases of envenoming, in the global perspective. In Nigeria, three snake species are primarily associated with envenoming, namely; the carpet viper (*E. ocellatus*), followed by puff adder (*B. arietans*) and the black-necked spitting cobra (*Naja nigricollis*) belonging to the *viperidae* and *elapidae* families respectively [3,7]. Proteomics approaches have been explored to comprehensively study complex proteins and their functions [8]. It also allows for the detection of minute differences in the venom of very closely related snake species [9], demystifying the complexity and diversity of snake venoms. One of the proteomic approaches used for unraveling the complexity of snake venoms, identification, and characterization of constituent venom proteins is two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry (MS) [10–12].

The evolution of proteomics technologies, particularly the 2-DE coupled with MS strategy that was successfully used for the analysis of snake venoms has provided promising insights into venom composition, protein function and evolutionary relationships in snakes [13]. The aim of this study was to comparatively analyze (inter species) venoms from four snake species belonging to the two most medically important snake families. Using 2-DE coupled with MALDI-TOF MS, we present the first comparative proteomic analysis of venoms from the *elapidae* (*N. haje* and *N. katiensis*) and *viperidae* (*E. ocellatus* and *B. arietans*) snake families native to Nigeria.

Methods

Snakes

Ahmadu Bello University approved all experimental protocols, and snake handling was approved and carried out in accordance with the Ahmadu Bello University Committee on Animal Use and Care. All methods are reported in accordance with ARRIVE guidelines 2.0 for animal experiment reporting [14]. During the rainy season, adult male snakes from the *viperidae* (*E. ocellatus* and *B. arietans*) and *elapidae* (*N. haje* and *N. katiensis*) families were randomly captured from different regions of Southern and Northern Nigeria. In each region, six snakes (three of each species) were captured. They were kept at the serpentarium, at the Department of Veterinary Pharmacology and Toxicology. Their venoms were manually extracted after three days, as described by Hill and Mackessy [15]. Snake venoms from the same species were combined. They were frozen at -80 °C, lyophilized in a freeze-dryer, and stored at -20 °C.

Venom protein extraction

The extraction of venom proteins was carried out as previously described [8]. Dry weights (2 mg) of the lyophilized venom were solubilized in 50 µL 1X PBS and centrifuged at 15,700 × *g* for 5 min at 4°C. Six hundred (600) µL of cold acetone was added to the supernatant and the samples were incubated for 15 min at -20°C. The protein pellets were air-dried and suspended in 100 µL 1X PBS buffer (pH 7.4). Protein concentration of each sample was quantified as described [16].

Two-dimensional gel electrophoresis

The protein samples were separated in a first dimension by isoelectric points using immobilized pH gradient (18 cm IPG) strips (pH 3-10) and further separated by molecular weights in a second dimension on 2-DE gels. Protein samples (120 µg) were premixed to a volume of 350 µl with DeStreak rehydration solution containing 2 % ampholytes and loaded onto a rehydration tray. The IPG strips were carefully placed on each sample, the surface of the strip was covered with mineral oil and allowed to rehydrate for 16 hours at 26°C. They were focused using isoelectric focusing (IEF) PROTEAN IGPHOR (Bio-Rad) instrument. After the isoelectric focusing, strips were placed in equilibration buffer containing SDS for solubilization of focused proteins and the binding of SDS for the second dimension separation. After equilibration and second dimension separation, the gels were stained with Colloidal Coomassie Blue. They were destained in 7% acetic acid, rinsed in deionized water and visualized.

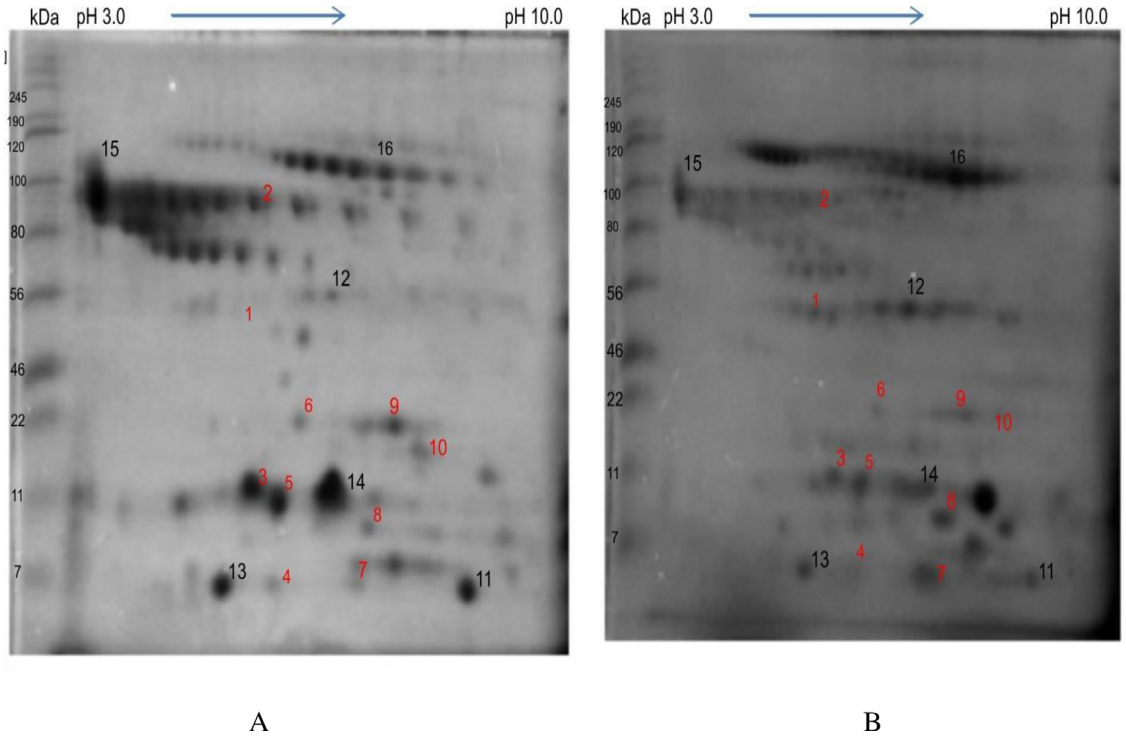


Figure 1. Two-dimensional electrophoresis (2-DE) of the venoms of (A) *N. haje* and (B) *N. katiensis*

Comparative analysis of 2-DE on the venom samples

Differential 2D protein spots ($p<0.05$) were selected and analyzed in three replicates per sample using PDQuest™ software version 8.0 (Bio-Rad). Imaging of the 2D gel was done using a Molecular Imager PharosFX Plus System (Bio-Rad). The gels were normalized using local regression model to provide a balance for gel to gel differences in spot quantities because of non-expression related variations [9]. For optimum spot detection the following parameters were selected: Sensitivity: 9, Minimum Peak: 500, Size scale: 3 and based on the Gaussian Model Test for statistical analysis of spots. Prior to protein detection, spots were selected and edited manually using a group consensus tool to obtain spot expression consensus across all biological replicates in samples. Protein spot with a *p*-value of less than 0.05 and a fold change of more than 2 was considered differentially present between the venom samples. Three biological replications were used for the analysis.

Matrix-assisted laser desorption and ionization time-of-flight mass spectrometer (MALDI-TOF MS) analysis

The Coomassie-stained 2-DE gels were scanned using Molecular Imager PharosFX Plus System (Bio-Rad). Protein spots of interest were destained, selected, excised and reduced using dithiothreitol and alkylated with the aid of iodoacetamide. The spots were dried using a SpeedVac® centrifugal evaporator (Thermo Scientific, USA) and suspended in 25 mM ammonium bicarbonate containing 10 µL of 10 ng/µL trypsin and incubated overnight at 37°C. Aliquots of the tryptic-digested spot samples were mixed with α-cyano-4-hydroxy cinnamic acid (Sigma) in 50% acetonitrile containing trifluoroacetic acid. The sample (0.5 µL) was spotted on a MALDI-TOF target plate, dried to crystallization and was analyzed by MALDI-TOF as previously described [17]. An Ultraflex-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) was used for this purpose. The Ion Sources 1 and 2 were respectively set at 25 kV and 21.9 kV, with a delay time of 20 ns. Calibration of the instrument was done with Bruker Standard Peptide Calibration kit (m/z 1000-3500).

Database search

The MALDI-TOF mass spectra of the peptides obtained were imported into Proteome Discoverer v1.4 software (Thermo Fisher Scientific, USA). Proteins were identified by a search against concatenated Uniprot-Serpentes database at https://www.thegpm.org/crap/. The search results were further validation at www.proteomesoftware.com. Peptide identification and matching to peptide sequence were performed using X! Tandem at 99% protein threshold, 95% peptide threshold, and two-peptide minimum criterion.

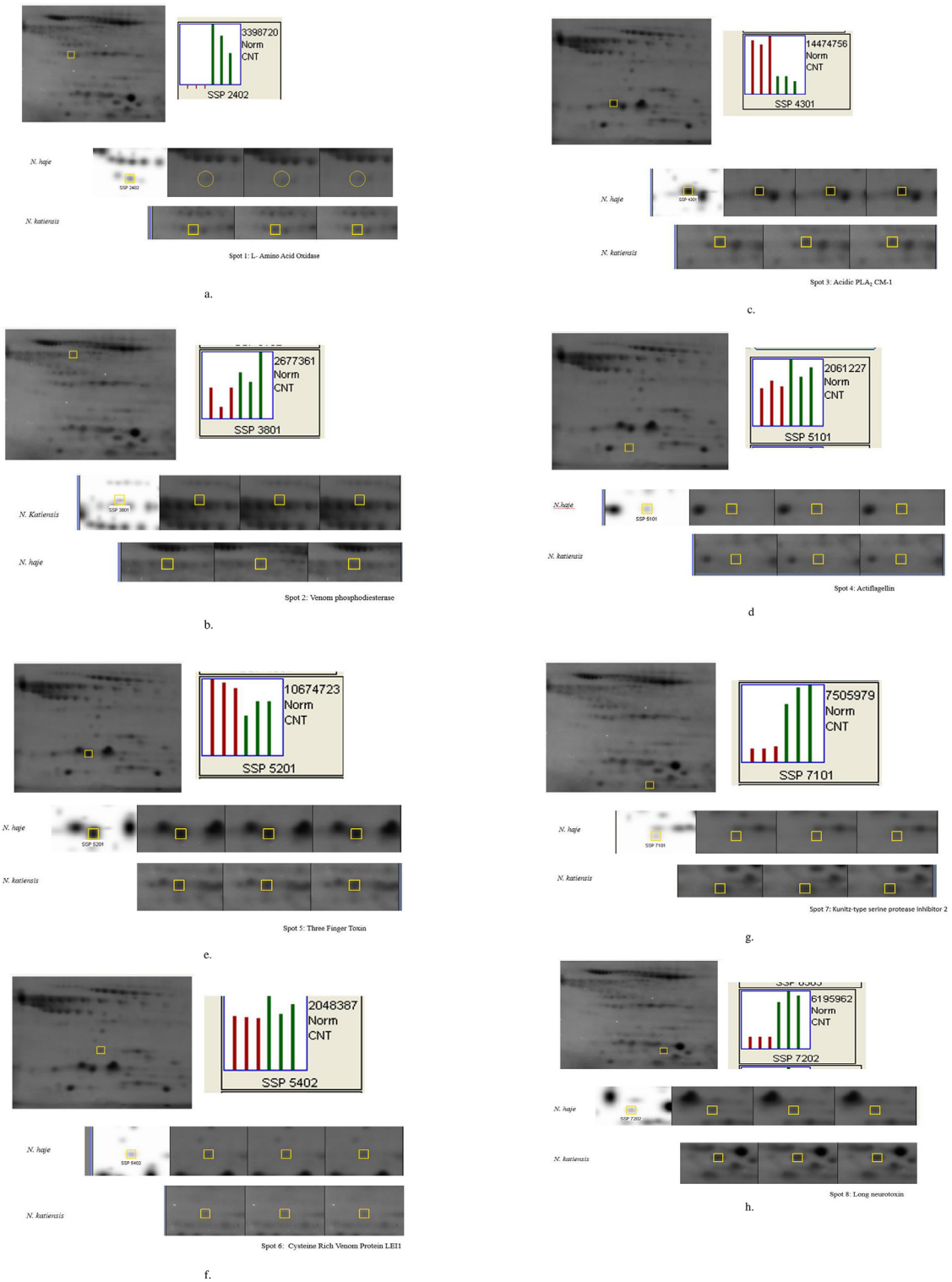


Figure 2. The PDQuest image showing differential expression for each of the selected and analyzed protein spots (a-h) from the venom proteome of *N. haje* and *N. katiensis*.

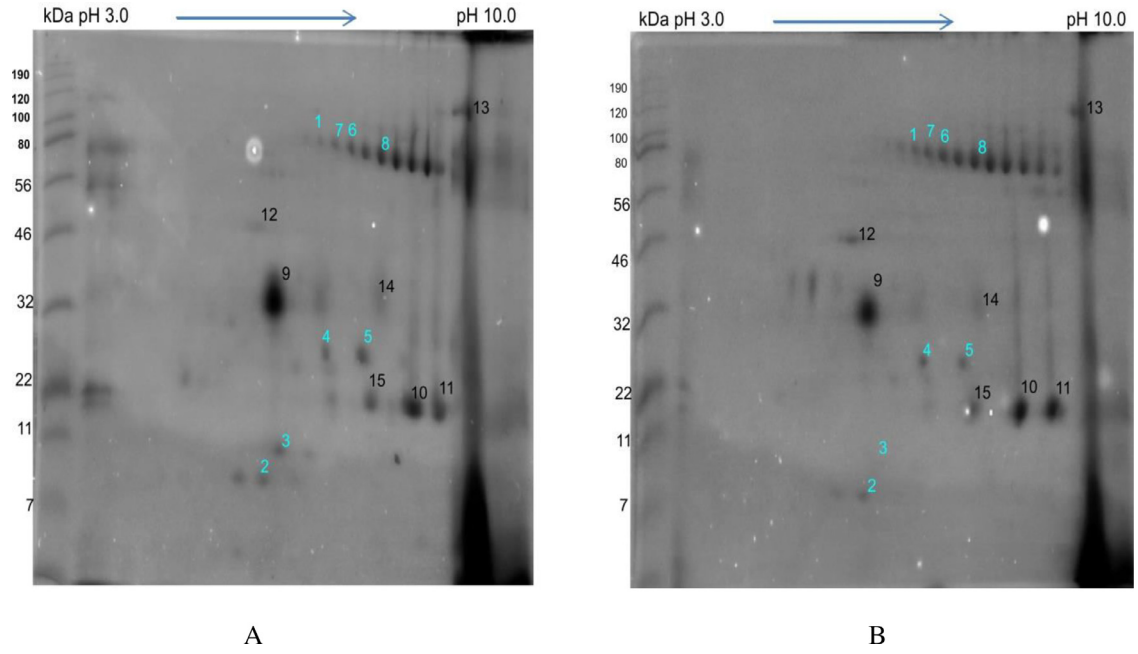


Figure 3. Two-dimensional electrophoresis (2-DE) of the venoms of (A) *B. arietans* and (B) *E. ocellatus*

Results and discussion

Despite the fact that two-dimensional gels have a limitation in adequately resolving complex samples, it was revealed that there is distinct variation in the toxin profile of snake venoms at both the species and family levels (Figure 1). Toxin composition differences between snake families and species are important considerations in antivenom production strategy, improved specificity, and efficacy of treatment outcome in envenomed patients [18–20].

*Venom comparative analysis of the elapidae (*N. haje *and* N. katiensis*) and viperidae (*B. arietans *and* E. ocellatus*) snakes*

The differential toxin abundance and composition of the venom from these snakes were obtained after the 2-DE gels were normalized using local regression model to account for gel to spot differences [9]. Acid proteins (low pI values) with a molecular mass range of 5 - 94 kDa predominated in the venoms of the elapids (*N. haje* and *N. katiensis*). The neurotoxin and Kunitz-type serine protease inhibitor had basic isoelectric points (pI), whereas venom phosphodiesterase, phospholipase A_2, three finger toxins, and cysteine-rich venom protein had acidic isoelectric points (pI) (Table 1). This is consistent with elapid snake venom, which is primarily composed of toxins with systemic neurotoxic effects such as neuromuscular paralysis [2,21–23]. Elapid snake venom contains a diverse range of toxin families, primarily phospholipase A_2 and three-finger toxins [8,24], which cause neurotoxic and myotoxic effects in victims [25–27].

In a parallel comparison, most of the proteins found in *viperid* venoms had pI in the range of 7, including snake venom metalloproteinase, L-amino acid oxidase, cytotoxin, snake venom serine protein, cysteine-rich venom protein, zinc metalloproteinase-disintegrin, disintegrin/ metalloproteinase domain-containing protein, and venom phosphodiesterase. They ranged in molecular mass from 4 to 95 Da (Table 2). Toxins in *viperid* snake venom cause local tissue damage and abnormal clinical manifestations [22,2], which can lead to permanent damage [2].

Differetial protein abundance

Toxins in the venom of the elapid snakes *N. haje* and *N. katiensis* differetially expressed in intraspecies comparison, indicating that despite belonging to the same family, variation exists in the venom composition of these snakes. The PDQuest annotated spots on the 2-DE gels revealed that L-amino acid oxidase was present in *N. katiensis* venom but absent in *N. haje* venom, as shown on spot 1 (Figure 2a), whereas venom phosphodiesterase was found to be more abundant in *N. haje* venom than *N. katiensis* venom, as shown on spot 2. (Figure 2b). The protein on spot 3 was identified as acidic phospholipase A_2, which was abundant in the venom of *N. haje* (red bar) compared to *N. katiensis* (green bar), as indicated by the thickness of the spots (Figure 2c). Actiflagelin, an uncommon protein, was found to be equally distributed in both venoms, as shown in Figure 4. (Figure 2d). In addition, three-finger toxin (3FTx) was more abundant in the venom of *N. haje* than *N. katiensis*, as shown in spot 5. (Figure 2e). Spots 6, 7, and 8 each represent a cysteine-rich venom protein, a kunitz-type serine protease inhibitor, and a neurotoxin (Figure 2f-h). These toxins were found in greater abundance in the venom of *N. katiensis* than

Table 1
Protein identification of PDQuest annotated spots in the venom proteomes of *N. haje* and *N. katiensis*

Venom sample spot *N. haje*	PDQuest annotated spot ID	Identified protein	Accession number	Molecular mass (kDa)	Isoelectric point (pI)	Sequence coverage (%)
1	SSP 2402	Absent				
2	SSP 3801	Venom Phosphodiesterase	PDE_NAJAT	94	5.63	19
3	SSP 4301	Acidic PLA_2 CM-1	PA2A1_NAJMO	13	5.37	27
4	SSP 5101	Actiflagelin	3NOJ_WALAE	7	5.78	14
5	SSP 5201	Three Finger Toxin W-V	3NO25_WALAE	9	5.90	15
6	SSP 5402	Cysteine Rich Venom Protein LEI1	CRVP_LEIMD	24	6.45	26
7	SSP 7101	Kunitz-type serine protease inhibitor 2	VKT2_HEMHA	5	8.03	15
8	SSP 7202	Long Neurotoxin OH-55	3L22_NAJME	7	9.11	11

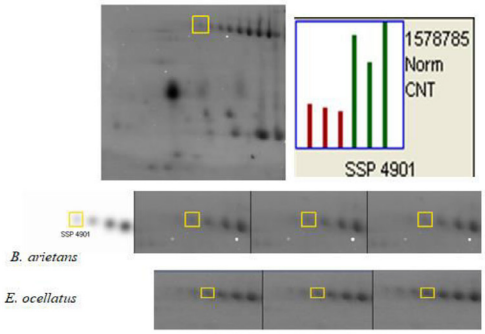
N. katiensis

1	SSP 2402	L-amino acid oxidase	OXLA_NAJAT	54	5.13	6
2	SSP 3801	Venom Phosphodiesterase	PDE_NAJAT	94	5.63	22
3	SSP 4301	Acidic PLA_2 CM-1	PA2A1_NAJMO	13	5.32	19
4	SSP 5101	Actiflagelin	3NOJ_WALAE	7	5.72	11.8
5	SSP 5201	Three Finger Toxin	3NO25_WALAE	9	6.20	13
6	SSP 5402	Cysteine Rich Venom Protein	CRVP_LEIMD	24	6.36	25
7	SSP 7101	Kunitz-type serine protease inhibitor 2	VKT2_HEMHA	6	8.32	31
8	SSP 7202	Long Neurotoxin OH-55	3L22_NAJME	7	9.01	24

Table 2
Protein identification of PDQuest annotated spots in venom proteomes of *B. arietans* and *E. ocellatus*

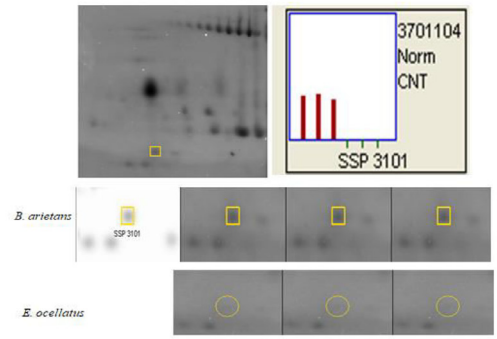
Venom sample spot *Bitis arietans*	PDQuest annotated spot ID	Identified protein	Accession number	Molecular mass (kDa)	Isoelectric point (pI)	Sequence coverage (%)
1	SSP 4901	Snake venom metalloproteinase-acutolysin-C	VM1AC-DEIAC	95	6.76	7
2	SSP 3101	L-amino-acid oxidase	OXLA_BITGA	7.8	6.25	12
3	SSP 3001	Cytotoxin 1	3SA1_NAJMO	7	6.65	58
4	SSP 5305	Snake venom serine protease HS114	VSP14_BOTJA	28	6.96	42
5	SSP 6303	Cysteine-rich vensom protein	CRVP_TRIST	26	7.42	18
6	SSP 6801	Zinc metalloproteinase-disintegrin BlatH1	VM2H1_BOTLA	54	7.86	4.1
7	SSP 6901	Disintegrin and metalloproteinase domain-containing protein 17	ADA17_HUMAN	93	7.23	0.97
8	SSP 9802	Venom phosphodiesterase	PDE_MACLB	96	9.24	7.9

Echis ocellatus						
1	SSP 4901	Zinc metalloproteinase-disintegrin-like stejnihagin-B	VM3SB_TRIST	68	5.96	12.3
2	SSP 3101	L-amino-acid oxidase	OXLA_BITGA	7	6.13	20
3	SSP 3001	Cytotoxin 1	3SA1_NAJMO	4	6.82	31
4	SSP 5305	Snake venom serine protease HS114	VSP14_BOTJA	28	7.06	17
5	SSP 6303	Cysteine-rich venom protein	CRVP_TRIST	26	7.42	24
6	SSP 6801	Zinc metalloproteinase-disintegrin BlatH1	VM2H1_BOTLA	54	6.96	2.5
7	SSP 6901	Disintegrin and metalloproteinase domain-containing protein 17	ADA17_HUMAN	93	7.47	9
8	SSP 9802	Venom phosphodiesterase	PDE_MACLB	9.25	7.84	9.8



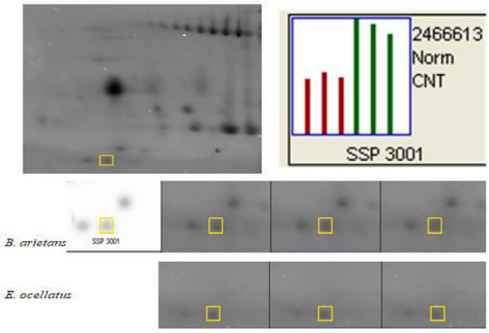
Spot 1: Snake venom metalloproteinase-acutolysin-C

a.



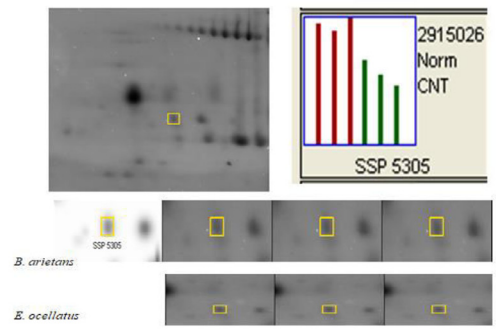
Spot 2: L-amino-acid oxidase

b.



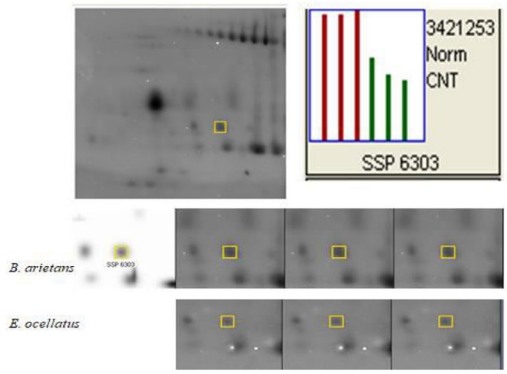
Spot 3: Cytotoxin 1

c.



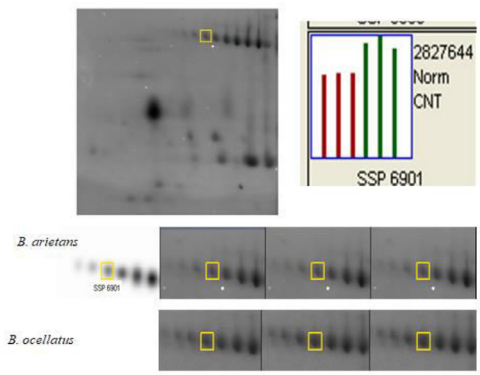
Spot 4: Snake venom serine protease HS114

d.



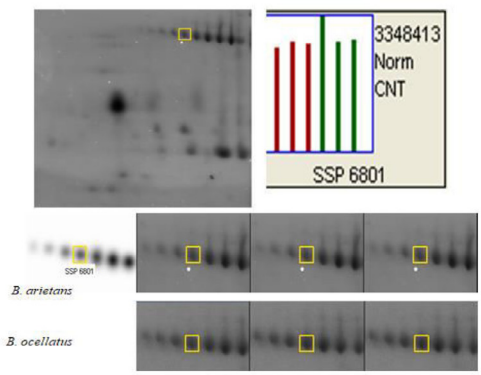
Spot 5: Cysteine-rich venom protein

e.



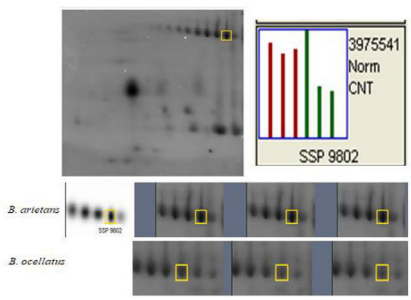
Spot 7: Disintegrin and metalloproteinase domain-containing protein 17

g.



Spot 6: Zinc metalloproteinase-disintegrin BlatH1

f



Spot 8: Venom phosphodiesterase

h.

Figure 4. The PDQuest image showing differential expression for each of the selected and analyzed protein spots (a-h) from the venom proteome of *B. arietans* and *E. ocellatus*.

in *N. haje*. In a parallel comparison, *N. katiensis* produced more toxins, implying that victims of *N. haje* envenoming may suffer more severe neurological effects leading to paralysis. Because of the complexity and diversity of venom composition, the clinical effect of envenomation varies significantly [2]. Uncovering the toxin variability in the analyzed venoms may aid in the development of effective antivenom with broad-spectrum activity as a road map for reducing the life-threatening impact of snakebite envenoming.

The 2-DE of venoms from the two viper snake species *B. arietans* and *E. ocellatus* (Figure 3) revealed significant differences in venom composition. The PDQuest annotated spots on the 2-DE gels revealed that snake venom metalloproteinase (SVMP) is more abundant in *E. ocellatus* venom than *B. arietans* venom, as shown on spot 1. The green and red bars represent the abundance of SVMP in *B. arietans* and *E. ocellatus* venom, respectively (Figure 4a). Envenomation by the viper is the leading cause of death in Nigeria, with *E. ocellatus* responsible for at least 66% of reported cases [3]. The high abundance of SVMP in *E. ocellatus* venom indicates that it produced more toxins, which could be attributed to its rapid lithality in envenomed patients [16]. SVMP is a zinc-dependent proteinase toxin that is a major component of viper venom [24] and a very lethal venom protein of viper snakes [28,29,30]. SVMP play an important role in the coagulopathies commonly associated with viper envenoming [28,31,30] and cause a wide range of clinical manifestations, including hemorrhagic, anticoagulant, fibrinolytic, and antiplatelet activity [32,33]. In spot 2, L-amino acid oxidase is expressed in *B. arietans* venom but absent in *E. ocellatus* venom (Figure 4b). The protein identified in spot 3 (Figure 4c) is cytotoxin, which is found in greater abundance in *E. ocellatus* venom than in *B. arietans* venom. As shown in Figure 4d, snake venom serine protease is more abundantly expressed in *B. arietans* venom than *E. ocellatus* venom. The same trend was observed for spot 5 cysteine-rich venom protein, which was more abundantly present in *B. arietans* venom than *E. ocellatus* venom (Figure 4e). Zinc metalloproteinase (spot f), Disintergrin/metalloproteinase domain-containing protein (spot g), and venom phosphodieasterase (spot h) were all found but expressed differently in both venoms (Figure 4f-h). Although the major toxins in the venom of the most medically important Nigerian viper and elapid snakes were identified, the large number of other protein spots from the two-dimensional electrophoresis gels that were not identified limits the scope of this study. Toxins found in viper snake venom included snake venom metalloproteinase, snake venom serine protease, and cytotoxins, while phospholipase A_2, three-finger toxins, and neurotoxins were found in elapid snake venom.

The protein contents of Nigerian snake venoms *N. haje* and *N. katiensis* [8,34], *E. ocellatus* [16,35], and *B. arietans* [16,36] have been studied. Toxins in venom, on the other hand, differ between and within species. As a result, understanding the variation pattern can help guide the development of new and better therapeutic strategies. The current study is an attempt to study variation by comparing the venoms of *N. haje* and *N. katiensis*, as well as *E. ocellatus* and *B. arietans.*

Conclusion

Viperid snake venoms contained cytotoxic-inducing proteins such as SVMPs, SVSPs, and cytotoxins, whereas elapid snake venoms contained neurotoxic proteins such as PLA_2, 3-FTx, and neurotoxins. Toxins in the analyzed venoms vary and are expressed differently in the venoms; therefore, taking this into account may allow for the development of novel and effective therapeutic strategies. Inter- and intra-venom toxin variation undermines the efficacy of the available polyvalent antivenom treatment. This confirms envenomation as a deadly tropical disease. Because of the observed variation, we hypothesize that tailored antivenom production can aid in the treatment of snakebite victims.

Concept for publication

Not applicable.

Ethics approval and concept to participate

The experimental protocols were approved by the Ahmadu Bello University Committee on Animal Use and Care and followed the revised ARRIVE guidelines 2.0.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conceptualization FAA, EJD, MSA and ABS. FAA, EJD GM, and AK designed and did the experiments. FAA and EJD analyzed the data and wrote the initial draft of the manuscript. AK and EJD revised the manuscript. ABS and MSA supervised the work. All authors critically reviewed and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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