

Article

Patterns in Protein Components Present in Rattlesnake Venom: A Meta-Analysis

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Abstract: The specificity and potency of venom components gives them a unique advantage in development of various pharmaceutical drugs. Though venom is a cocktail of proteins rarely is the synergy and association between various venom components studied. Understanding the relationship between various components is critical in medical research. Using meta-analysis, we found underlying patterns and associations in the appearance of the toxin families. For *Crotalus*, Dis has the most associations with the following toxins: PDE; BPP; CRL; CRiSP; LAAO; SVMP P-I & LAAO; SVMP P-III and LAAO. In *Sistrurus* venom CTL and NGF had most associations. These associations can be used to predict presence of proteins in novel venom and to understand synergies between venom components for enhanced bioactivity. Using this approach, the need to revisit classification of proteins as major components or minor components is highlighted. The revised classification of venom components needs to be based on ubiquity, bioactivity, number of associations and synergies. The revised classification will help in increased research on venom components such as NGF which have high medical importance.

Keywords: Rattlesnake; *Crotalus*; *Sistrurus*; Venom; Toxin; Association

Key Contribution: This article explores the patterns of appearance of venom components of two rattlesnake genera: *Crotalus* and *Sistrurus* to determine the associations between toxin families. This will help us understand the synergistic activities between these components. Synergistic relationship between toxins can be exploited in development of new pharmaceutical drugs.

1. Introduction

Venom has become an integral part of the biomedical research[1]. Various venom components have been critical in development new pharmaceutical drugs[2], and possible treatment of diabetes, strokes, heart attacks[3,4], and cancer[5-12]. Venom for most of these researches are sourced from various venomous organisms such as snakes, scorpions, spiders etc. Within snakes, venomous snakes are mainly in three families: *Atractaspidae*, *Elapidae*, *Viperidae*[13]. Despite being just three families the complexity and variability in the venom composition is immense, often being described as a venom cocktail[14-16]. The variation in biochemical composition of snake venom can occur within closely related species and also within species itself[1,17-22]. For example, intra-genus or intra-specific variation in venom in pit vipers and adders [17,23] has been correlated to diet [17,18,24,25] or topographical features[26,27]. One of the primary reasons for high diversity and plasticity in snake venom is due to frequent duplication of toxin-encoding genes and recruitment strategy[28-32]

followed by functional and structural diversification [1,33-37]. The accelerated rate of venom diversification is supported by the hypothesis that suggests the use of venom for predation [17,33,38-40] and prey digestion supports[17].

Within the North and South American continent, it is suggested that venom of *Crotalidae* has the highest variation in toxicity with high proteolytic activity[41]. Rattlesnakes are within subfamily *Crotalinae*, of two genera *Crotalus* and *Sistrurus*. They are native to the Americas ranging from southern Alberta, Saskatchewan, and southern British Columbia in Canada to central Argentina. There are approximately 32 species of rattlesnakes within the *Crotalus* and *Sistrurus* genus[42]. These snakes are found in a myriad of habitat types ranging from the Sonoran Desert of northwestern Mexico to alpine and cloud forest in central and southern Mexico[42]. They occur from below sea level in desert basins in California to about 4,500 m in the Transverse Volcanic Cordillera of central Mexico[42]. Mexican Plateau and its fringing mountains have the highest diversity of rattlesnakes [42]. This high variability in the habitat type, altitude, and associated diet types along with a large geographical range allows the rattlesnakes to have high variability in their venom composition.

Rattlesnakes possess a variety of different toxins from 10-20 protein families [7,30,43-45] and may not possess all of them at once. These families are enzymatic proteins such as L-amino Acid Oxidases (LAAO) [30,46-49], Phosphodiesterase (PDE)[50-52], Snake Venom Metalloproteases (SVMP)[53-55], Serine Proteases (SVSP)[47,56-58], Phospholipases (PLA₂)[59-62]; or non-enzymatic proteins like Myotoxin a and its homologs[63-66], Bradykinin-Potentiating Peptides and Bradykinin-Inhibitory Peptide (BPPs & BIPs)[30,47,67,68], Disintegrins (Dis)[3,47,48,58,69-71], Cysteine-Rich Secretory Proteins (CRiSPs)[2,48,58,65], and C-Type Lectins (CTL)[30,44,51,72]. It is not uncommon for variation in venom composition within species[17,23]. This plasticity and variability of venom gives it a unique advantage in biomedical research.

Despite venom being of high importance in medical research, many problems in researching rattlesnake venoms still persist: lack of venom composition studies for several species, often the rare and geographically inaccessible ones, within the genus *Crotalus*; high cost associated with venom-based studies; high variability in venom composition rendering venom composition studies for all age classes in all populations of rattlesnakes impractical; and sparse data on relationship between various venom components makes it impossible to predict the venom components in any species. This dilemma can be avoided by studying relationship between venom components rather than individual units. For example, within *Crotalus polystictus*, type and potency of proteins expressed varies with age and sex [51,72-74]. Many venom components discovered, such as LAAO and PDE, have not been explored for possible biomedical applications based on major databases searches[32,75]. However, based on the associations and ubiquity alone, it is evident that they do play a role during envenomation.

These new-found relationships between various protein components in snake venom may play key roles in developing suitable treatments for prevalent diseases. Such relationships between toxins, often termed synergisms, are joint effects of multiple toxins which assert greater effects than sum of individual potencies, thus allows snake venoms to be effective with only a trace amounts of venoms[76,77]. In the current study we conduct a meta-analysis to understand the relationship between various of protein components in *Crotalus* and *Sistrurus*' venoms. We also report the frequency with which various proteins are present in *Crotalus* and *Sistrurus*' venoms. Using the

medical relevance, relationship to different protein type and frequency of protein appearance can we classify proteins as major component or minor component in venom.

2. Result

Out of 192 studies that were screened, 36 studies that did not meet inclusion criteria and 41 studies that met the exclusion criteria were excluded. Remaining 115 full-text articles are included in the analysis.

2.1. Venom constituents in *Crotalus* venom

We identified compositional venom studies, through both transcriptomic and proteomic technologies, for 30 entries, including species and subspecies, within the genus *Crotalus*. 46 protein families were present in *Crotalus* (Table 1). There was no study found regarding the venom composition of nine *Crotalus* species and sub-species. These protein families could be classified based on ubiquity or relationship with other proteins.

Table 1. Venom components within *Crotalus* genus

Species	Venom components	Reference
<i>C. adamanteus</i>	5'NT, BPP, Carboxypeptidase (E-Like), CNP, CRiSP, CTL, Dipeptidase, Dis, EF-hand protein, EGF, GC, Hya, Kun, LAAO, MYO, NGF, PDE, PLA ₂ , PLB, SVMP-P I/II/III, SVSP, VEGF, Vespryn	[78-90]
<i>C. aquilus</i>	Hya, PLA ₂ , SVMP P-III, SVSP (TLE)	[62,72]
<i>C. atrox</i>	BIPs, BPPs, CNP, CRiSP, Dis, Hya, LAAO, CTL, PLA ₂ , SVMP P-I/III, SVSP, VEGF	[6,47,83,91-98]
<i>C. basiliscus</i>	BPP, CRISP, CTL, Dis, LAAO, PLA ₂ (CRTX, non-CRTX), SVMP P-I/II/III, SVMP-inhibitor, SVSP	[99-102]
<i>C. catalinesis</i>	SVSP, SVMP P-III, PLA ₂	[43]
<i>C. cerastes</i>	3FTx, 5'NT, BPP, CRiSP, CTL, Dis, Ficolin, Hya, Kun, LAAO, MYO, NGF, PDE, PLA ₂ , SVMP P-II/III, SVSP, VEGF, Vespryn, WAP	[101,103-105]
<i>C. durissus</i>	3FTx, Achase, Aminopeptidase, Angiogenin, BPP, Carboxypeptidase, CNP, CRiSP, CTL, CysProt inhibitor, CysProt, Dipeptidyl Peptidase, Dis, FGF, Fraction 5, Hya, Kazal, Kun, LAAO, Lipase, MYO, NGF, PDGF, PLA ₂ (non-CRTX, CRTX), PLB, PLD, Serpin-like, SVMP inhibitor, SVMP P-III, SVSP, VEGF, Vespryn, WAP	[48,67,106-121]
<i>C. enyo</i>	SVSP, SVMP P-I/III, PLA ₂	[43]
<i>C. horridus</i>	5'-NT, BPP, CNP, CRiSP, Dis, EGF-like, GC, Hya, Kun, LAAO, MYO, Neurotrophic Factor, NGF, PDE, PLA ₂ , SVMP P-I/III, SVSP, VEGF, Vespryn	[29,122,123]
<i>C. lepidus</i>	5'NT, CRiSP, CTL, Dis, LAAO, PDE, PLA ₂ , SVMP-P-I/III, SVSP (TLE, Kallikrein)	[52,124-126]
<i>C. mitchelli</i>	LAAO, SVSP, PLA ₂ (CRTX/MTX)	[43,127,128]
<i>C. molossus</i>	Dis, LAAO, MYO, PLA ₂ , SVMP P-I/III, SVSP (TLE)	[61,101,129-133]

<i>C. oreganus</i>	ANP/BNP, BPP, CNP, CRiSP, CTL, Dis, Hya, Kun, LAAO, MYO, NGF, PLA ₂ (D49), PLA ₂ , SVMP P-II/III, SVSP, VEGF, Vespryn	[54,59,60,134-140]
<i>C. polystictus</i>	BIPs, CRiSPs, CTL, Dis, GC, Hya, LAAO, NGF, PDE, PLA ₂ , PLB, SVMP P-I/II/III, SVSP (Kallikrein, TLE), Vespryn	[51,72]
<i>C. ruber</i>	CTL, Dis, LAAO, PDE, PLA ₂ , SVMP P-I/III, SVSP (Kallikrein)	[43,50,141-146]
<i>C. scutulatus</i>	5'-NT, APase, BPPs, CRiSP, CTL, Dis, Hya, Kun, LAAO, MYO, NGF, PDE, PLA ₂ (MTX, non-CRTX), SVMP P-I/II/III, SVSP, VEGF, Vespryn	[44,55,66,147-151]
<i>C. simus</i>	3FTX, 5'-NT, BIPs, BPPs, CRiSP, CTL, Dis, GC, Hya, Kaz, Kun, LAAO, MYO, NGF, OHA, PDE, PLA ₂ (CRTX, non-CRTX), PLB, SVMP P-I/III, SVSP, VEGF, WAP	[7,57,71,152-154]
<i>C. tigris</i>	CRiSP, Dis, PLA ₂ (MTX), SVMP P-III, SVSP, VEGF	[58,155-157]
<i>C. vegrandis</i>	5'-NT, ATPase, BIP, BPP, Carboxypeptidase, CNP, CRiSP, CTL, Dis, Endonuclease (DNase, RNase), Exendin4-like Protein, Glutathione peroxidase, Hya, LAAO, MYO, NGF, PDE, PLA ₂ (CRTX), PLB, SVMP P-II/III, SVSP	[80,158-162]
<i>C. viridis</i>	5'-NT, APase, BPP, CRiSP, CTL, Dis, GC, LAAO, MYO, OHA, PDE, PLA ₂ (CRTX, non-CRTX), PLB, SVMP inhibitor, SVMP P-I/II/III, SVSP (TLE, Kallikrein)	[45,63-65,163-166]
<i>C. willardi</i>	CRiSP, CTL, Dis, LAAO, PDE, PLA ₂ , SVMP P-I/III, SVSP (TLE, Kallikrein)	[52,156]
<i>C. tortugensis</i>	N/A	
<i>C. stejneri</i>	N/A	
<i>C. tancitarensis</i>	N/A	
<i>C. lannomi</i>	N/A	
<i>C. pusillus</i>	N/A	
<i>C. transversus</i>	N/A	
<i>C. triseriatus</i>	N/A	
<i>C. unicolor</i>	N/A	
<i>C. intermedius</i>	N/A	

Note: Three-finger toxin (3FTx), 5'-nucleotidase (5'-NT), Acetylcholinesterase (AChase), Natriuretic peptide type A (ANP), Adenosine triphosphatase (ATPase), Bradykinin inhibitory peptide (BIP), Natriuretic peptide type B (BNP), Bradykinin potentiate peptide (BPP), C-type Lectins (CTL), Natriuretic peptide type C (CNP), Cysteine Protease (CysProt), Cysteine-rich secretory protein(CRiSP), Crotoxin (CRTX), Disintegrin (Dis), Epidermal growth factor (EGF), Fibroblast growth factor (FGF), Guanylyl cyclase (GC), Hyaluronidase (Hya), Kazal-type inhibitor (Kazal), Kunitz-type inhibitor (Kun), L-amino acid oxidase (LAAO), Mojave toxin (MTX), Myotoxin (MYO), Nerve growth factor (NGF), Ohanin (OHA), Phosphodiesterase (PDE), Platelet derive growth factor (PDGF), Phospholipase A₂ (PLA₂), Phospholipase B (PLB), Phospholipase D (PLD), Snake venom metalloprotease (SVMP), Snake venom serine protease (SVSP), Thrombin-like enzyme (TLE), Vascular endothelial growth factor (VEGF), Waparin (WAP).

2.1.1. Frequency of protein components in *Crotalus* venom

The ubiquitous protein families in *Crotalus* venom were: PLA₂, SVSP, SVMP P-III, Dis, LAAO, CRiSP, CTL, SVMP P-I, BPP, Hya, and PDE (Figure 1).

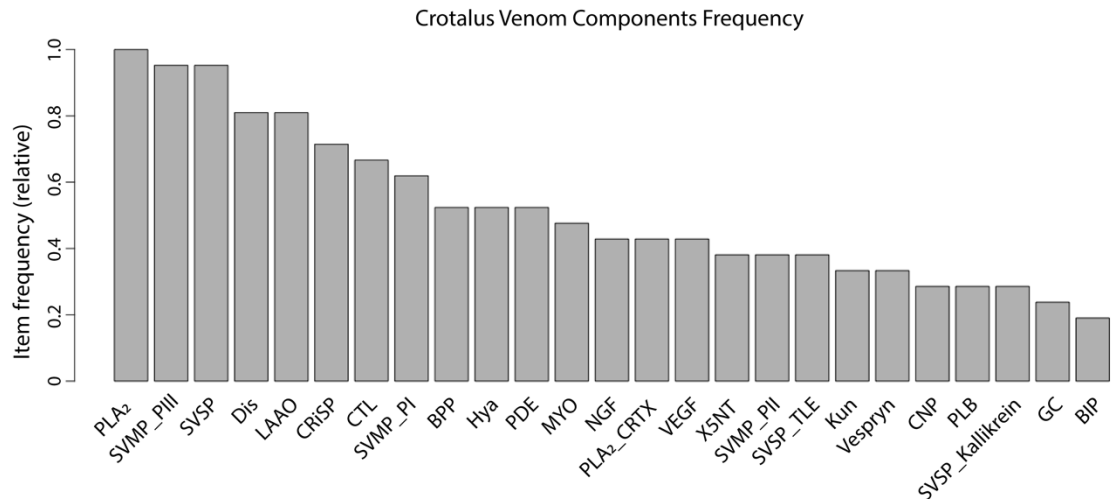


Figure 1. Twenty most common venom components in venom expressed by genus: *Crotalus*. PLA₂ and SVSP are identified as the most common amongst *Crotalus* species (relative frequency is 1). *Note:* 5'-nucleotidase (5'-NT), Bradykinin inhibitory peptide (BIP), Bradykinin potentiate peptide (BPP), C-type Lectins (CTL), Natriuretic peptide type C (CNP), Crotoxin (CRTX), Disintegrin (Dis), Guanylyl cyclase (GC), Hyaluronidase (Hya), Kunitz-type inhibitor (Kun), L-amino acid oxidase (LAAO), Myotoxin (MYO), Nerve growth factor (NGF), Phosphodiesterase (PDE), Phospholipase A₂ (PLA₂), Phospholipase B (PLB), Snake venom metalloprotease (SVMP), Snake venom serine protease (SVSP), Vascular endothelial growth factor (VEGF).

2.1.2. Association between various venom components in *Crotalus* venom.

Using the frequent item-set data mining, we were able to identify total of 559 relationships between different venom components for *Crotalus* (Table S1) (first three rules did not identify the predictor protein and hence were discarded). In this study we highlight the top 20 associations (Figure 2), e.g., Dis was associated with CTL with confidence =1, support = 0.667 (Table 2). Dis and CTL were expressed together 66.7% times in venom of all species of *Crotalus*, and if CTL was expressed in venom, then 100% times Dis was also expressed.

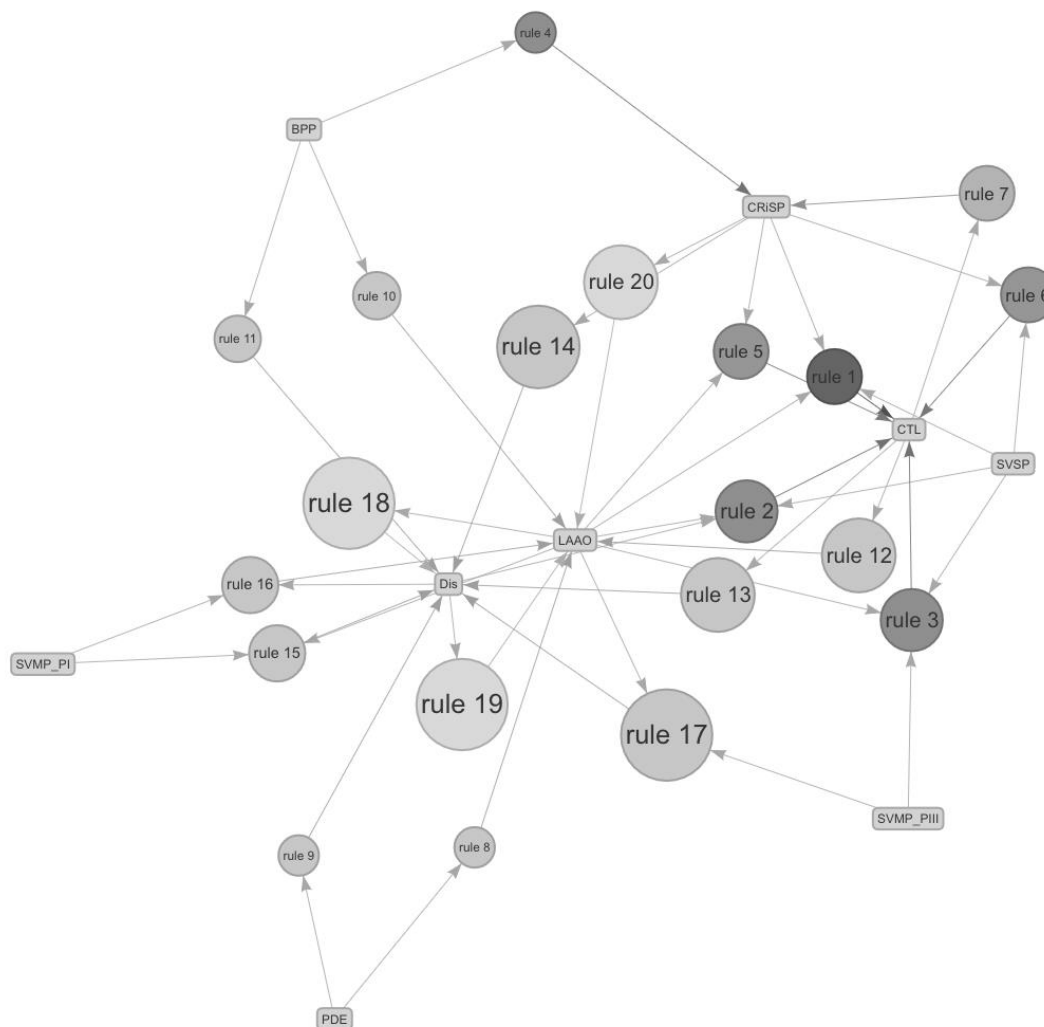


Figure 2. Depictions of association between components of venom expressed by genus *Crotalus*. The rules are depicted by top twenty minor components-related rules as stated in Table 2.

Table 2. Depictions of association rules between proteins expressed in *Crotalus* venom.

Rule no.	Protein (Predictor)	Protein (Predicted)	Support	Confidence	Lift
[1]	CRiSP, LAAO, SVSP	CTL	0.61904762	1	1.5
[2]	Dis, LAAO, SVSP	CTL	0.66666667	0.93333333	1.4
[3]	LAAO, SVMP P-III, SVSP	CTL	0.66666667	0.93333333	1.4
[4]	BPP	CRiSP	0.52380952	1	1.4
[5]	CRiSP, LAAO	CTL	0.61904762	0.92857143	1.39285714
[6]	CRiSP, SVSP	CTL	0.61904762	0.92857143	1.39285714
[7]	CTL	CRiSP	0.61904762	0.92857143	1.3
[8]	PDE	LAAO	0.52380952	1	1.23529412
[9]	PDE	Dis	0.52380952	1	1.23529412

[10]	BPP	LAAO	0.52380952	1	1.23529412
[11]	BPP	Dis	0.52380952	1	1.23529412
[12]	CTL	LAAO	0.66666667	1	1.23529412
[13]	CTL	Dis	0.66666667	1	1.23529412
[14]	CRiSP	Dis	0.71428571	1	1.23529412
[15]	LAAO, SVMP P-I	Dis	0.57142857	1	1.23529412
[16]	Dis, SVMP P-I	LAAO	0.57142857	1	1.23529412
[17]	LAAO, SVMP P-III	Dis	0.76190476	1	1.23529412
[18]	LAAO	Dis	0.76190476	0.94117647	1.16262976
[19]	Dis	LAAO	0.76190476	0.94117647	1.16262976
[20]	CRiSP	LAAO	0.66666667	0.93333333	1.15294118

Note: 5'-nucleotidase (5'-NT), Bradykinin potentiate peptide (BPP), C-type Lectins (CTL), Cysteine-rich secretory protein(CRiSP), Disintegrin (Dis), L-amino acid oxidase (LAAO), Nerve growth factor (NGF), Phosphodiesterase (PDE), Phospholipase A₂ (PLA₂), Snake venom metalloprotease (SVMP), Snake venom serine protease (SVSP).

Crotalus' venom components are studied well enough to generate more than 500 associations, but only the top twenty relevant rules with at least 1 minor components are depicted in Table 2. If Protein (Predictor) is present in venom, then chances of the protein (predicted) to be expressed in the venom is given by combination of confidence and lift. Dis has the highest amount of associations as a predicted component, which is 7: PDE, BPP, CRL, CRiSP, LAAO, SVMP P-I & LAAO, SVMP P-III & LAAO. Followed by LAAO with 6 associations: PDE, BPP, CTL, Dis & SVMP P-I, Dis and CRiSP. On the other hand, CTL is associated with 5 groups and CRiSP represents with 2 associations. However, 5 associations of CTL have higher lifter and confidence than LAAO's and Dis' indicating better associations.

2.2. Venom constituents in *Sistrurus* venom

We identified compositional venom studies, through both transcriptomic and proteomic technologies, for 34 entries, including species and subspecies, within the genus *Sistrurus*. Few studies have focused on the *Sistrurus* subspecies' venom. 19 protein families were present in *Sistrurus* (Table 3). These protein families could be classified based on ubiquity or relationship with other proteins.

Table 3. Venom components within *Sistrurus* genus

Species	Venom components	Reference
<i>S. catenatus</i>	3FTx, 5'-NT, BPP, CNP, CRiSP, CTL, Dis, GC, LAAO, MYO, NGF, PDE, PLA ₂ (CRTX, non-CRTX), PLB, Renin-like Aspartic Protease, SVMP P-I/II/III, SVSP, VEGF	[29,73,167-172]
<i>S. miliarius miliarius</i>	BPP, CRiSP, CTL, Dis, NGF, PLA ₂ , SVMP P-I/III, SVMP-inhibitor, SVSP	[74]
<i>S. miliarius streckeri</i>	BPP, CRiSP, CTL, Dis, NGF, PLA ₂ , SVMP P-I/III, SVMP-inhibitor, SVSP	[74]

<i>S. miliarius</i>	BPP, CNP, CRiSP, Dis, PLA ₂ , SVMP P-I/III, SVSP	[29,30,73,74,172-175]
<i>barbouri</i>		

Note: Three-finger toxin (3FTx), 5'-nucleotidase (5'-NT), Bradykinin potentiate peptide (BPP), C-type Lectins (CTL), Natriuretic peptide type C (CNP), Cysteine-rich secretory protein(CRiSP), Crotoxin (CRTX), Disintegrin (Dis), Guanylyl Cyclase (GC), Hyaluronidase (Hya), L-amino acid oxidase (LAAO), Myotoxin (MYO), Nerve growth factor (NGF), Phosphodiesterase (PDE), Phospholipase A₂ (PLA₂), Phospholipase B (PLB), Snake venom metalloprotease (SVMP), Snake venom serine protease (SVSP), Vascular endothelial growth factor (VEGF).

2.2.1 Frequency of protein components in *Sistrurus* venom

The dominant protein families based on ubiquity in *Sistrurus* were: BPP, CRiSP, Dis, SVMP, CTL, NGF, PLA₂, and SVSP (Figure 3). The main difference between *Crotalus* and *Sistrurus* proteins was due to the absence of 27 venom components in *Sistrurus* (Figure 1, 3). Some of the absent venom components from *Sistrurus*' proteomic and transcriptomic were: Alkaline phosphomonoesterase (APase), Acetylcholinesterase (Achase), Aminopeptidase, Angiogenin, Natriuretic peptide (ANP and BNP), ATPase, Bradykinin inhibitory peptide (BIP), Platelet-derived growth factor (PDGF), Carboxypeptidase, Cysteine Protease (CysProt) and CysProt inhibitor, Dipeptidase, Dipeptidyl peptidase, EF Hand Protein, Epidermal growth factor (EGF), Exendin4-like protein, Endonuclease (DNase and RNase), Fibroblast growth factor (FGF), Ficolin/Veficolin, Glutathione peroxidase, Hyaluronidase (Hya), Kazal-type inhibitor (Kazal), Kunitz-type inhibitor (Kun), Lipase, Ohanin (OHA), Platelet derive growth factor (PDGF), Vespryn, Phospholipase D (PLD), and Waparin (WAP).

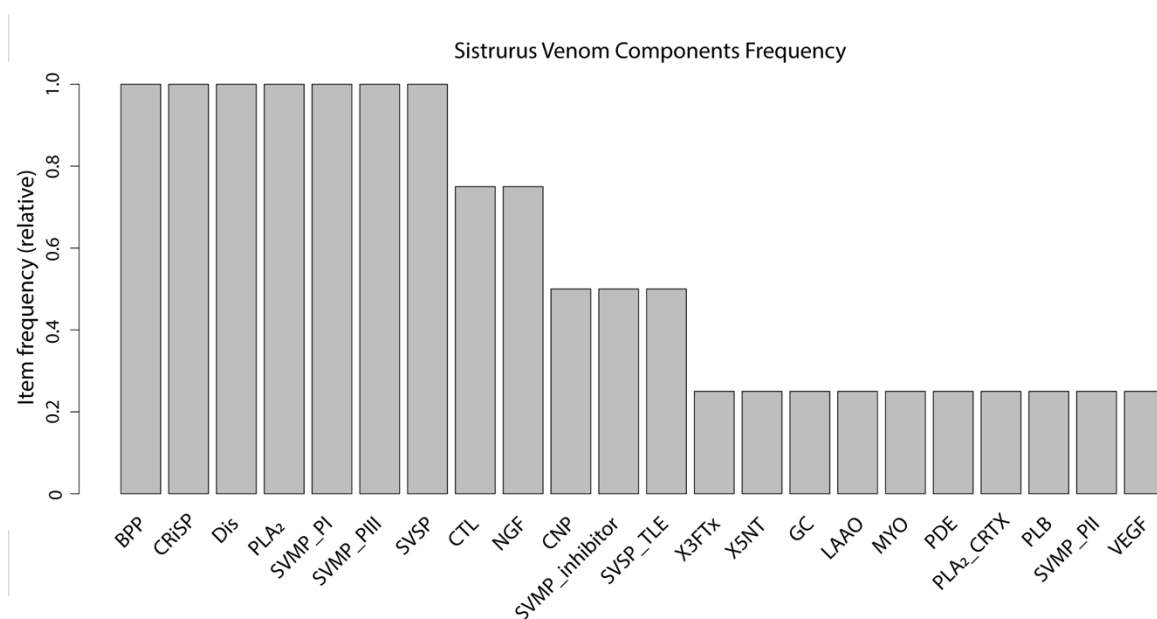


Figure 3. Twenty most common venom components in venom expressed by genus: *Sistrurus*. BPP, CRiSP, Dis, SVMP P-I/III are the most common toxins (relative frequencies are 1). Note: Three-finger toxin (3FTx), 5'-nucleotidase (5'-NT), Bradykinin potentiate peptide (BPP), C-type Lectins (CTL), Natriuretic peptide type C (CNP), Cysteine-rich secretory protein(CRiSP), Crotoxin (CRTX), Disintegrin (Dis), Guanylyl Cyclase (GC), L-amino acid oxidase (LAAO), Myotoxin (MYO), Nerve growth factor (NGF), Phosphodiesterase (PDE), Phospholipase A₂ (PLA₂), Phospholipase B (PLB), Snake venom metalloprotease (SVMP), Snake venom serine protease (SVSP), Vascular endothelial growth factor (VEGF).

2.2.2. Association between various venom components in *Sistrurus* venom.

Using the frequent item-set data mining, we were able to identify eight relationship between different venom components for *Sistrurus* (Figure 4), e.g., NGF was associated with CTL with confidence =1, support = 0.75 (Table 4). NGF and CTL were expressed together 75% times in venom of all species of *Sistrurus*, and if NGF was expressed in venom, then 100% times CTL was also expressed.

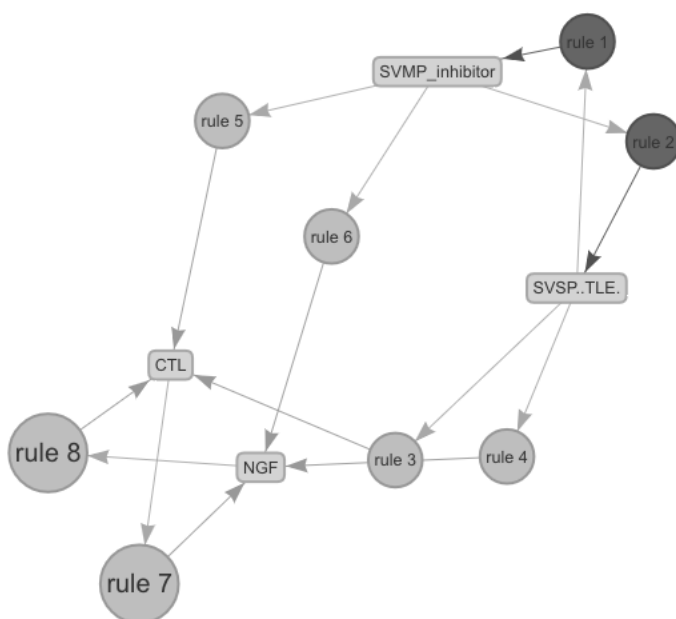


Figure 4. Depictions of association between components of venom expressed by genus *Sistrurus*. The rules are depicted by top twenty rules as stated in Table 2.

Table 4. Depictions of association rules between proteins expressed in *Sistrurus* venom.

Rules no.	Protein (Predictor)	Protein (Predicted)	Support	Confidence	Lift
[1]	SVSP	SVMP inhibitor	0.5	1	2
[2]	SVMP inhibitor	SVSP	0.5	1	2
[3]	SVSP	CTL	0.5	1	1.33333333
[4]	SVSP	NGF	0.5	1	1.33333333
[5]	SVMP inhibitor	CTL	0.5	1	1.33333333
[6]	SVMP inhibitor	NGF	0.5	1	1.33333333
[7]	CTL	NGF	0.75	1	1.33333333
[8]	NGF	CTL	0.75	1	1.33333333

Note: C-type Lectins (CTL), Nerve growth factor (NGF), Snake venom metalloprotease (SVMP), Snake venom metalloprotease inhibitor (SVMP inhibitor), Snake venom serine protease (SVSP).

In contrast to *Crotalus*' venom components, studies on *Sistrurus*' venom component are lacking and thus, only a small pool of studies were used to generate only 8 associations as depicted in Table 4. Similar to *Crotalus*' analysis, in this analysis if protein (Predictor) is present in venom, then

chances of the protein (Predicted) to be expressed in the venom is given by combination of confidence and lift. Both CTL and NGF have the highest amount of associations as a predicted component, which is 3: CTL is associated with NGF, SVMP inhibitor and SVSP; NGF is associated with CTL, SVMP inhibitor and SVSP. Followed by SVMP inhibitor and SVSP with 1 association each: SVMP inhibitor is associated with SVSP and vice versa. However, SVMP inhibitor and SVSP's associations have higher lift and confidence than CTL's and NGF's, indicating better associations.

3. Discussion

A total of 46 families of proteins were identified in the venom of 34 species and subspecies of rattlesnakes. The majority of studies have focused on *Crotalus*, and a subset of studies have focused on *Sistrurus*. Through our analysis we were able to discover a total of 562 rules for *Crotalus* and 25 rules for *Sistrurus* venom components. In this study we present 20 most relevant rules for *Crotalus* and eight rules for *Sistrurus* venom components, respectively. There's an emphasis on investigating venom components standalone units with a lack of investigations of their relationships with each other and the subsequent effects of co-administering different components. On the other hand, understanding the relationship between venom components could open a new avenue for biomedical research and unlock protein combinations that yield enhanced bioactivity in pharmaceutical drugs. Additionally, studying components as standalone may have produced a negative effect in which many components have received a skewed attention in biomedical research. For example, protein families are often classified as major or minor based on importance and ubiquity [13,131]. Thus, causing the dominant protein families such as Proteases, Neurotoxins, and Phospholipases to enjoy more attention than other protein families such as Growth Factors. However, it is by the combination of ubiquity, bioactivity, and relationship between the protein families that we can classify the venom components as major or minor.

In rattlesnakes MYO, PLA₂, SVMP and SVSP are classified as major components based on medical importance and ubiquity [13,131], which is also confirmed by our analysis (Figure 1). However, with a new approach of using both ubiquity and number of associations for each protein, we find that Dis, LAAO, CTL were all more ubiquitous and had more associations with other proteins in *Crotalus* species (Table 2). Similarly, in *Sistrurus* species, the SVMP inhibitor and NGF (Figure 4) have most associations as compared to MYO which has only one association (Table 3).

The few studies that have investigated venom proteins based upon their associations have highlighted the synergisms between toxins [76]. This synergism causes the joint effects of multiple toxins to assert greater effects than sum of individual potencies[76], making trace amount of snake venom to be highly efficient and effective [76,77]. Often, such combinations of venom proteins cause a variety of symptoms of bleedings, tissue degradation, necrosis, and further complications in prey and bite victims [72,176] and improve the lethality of whole crude venom in contrast to individual components[76,177].

Through mostly studies of predominant toxins, different general mechanisms for toxin synergisms have been proposed [76,178]:

- 1) Two or more toxins interacts with different targets on related biological pathways, resulting in synergistically increased toxicity
- 2) Two or more toxins recognize and interact with the same target in synergistic manner and produce the same effect, and is often called amplification

- 3) One toxin (subunit) acts as chaperone to potentiate another one. The chaperone may expose active/functional site of the 2nd toxin (subunit), or expose target sites, or increase affinity to target, or modify active surface of the other toxin (subunit). Such complexes usually dissociate after asserting their toxicity.

Synergisms has mostly been reported in major toxins in rattlesnake venoms[76]. Notable example of synergism through complex formation (mechanism 3) is crotoxin, a lethal neurotoxin from *C. durissus terrificus*, by 2 subunits: an acidic subunit component A (CA or crotapotin) and a basic subunit component B (CB) [112,118,119,179,180]. CB is identified as a basic PLA₂ with phospholipase activities and low toxicity while the CA component is said to be small acidic, nonenzymatic, nontoxic subunit[76,180]. However, once combine non-covalently, CA improves the potency of CB by enabling CB to reach the specific crotoxin receptors at the neuromuscular junction as well as inhibits other CB functions such as catalytic and anticoagulant activities[118,180]. Thus, the resulting crotoxin complex is highly active, comparing to individual components, and showing the synergistic between 2 subunits at blocking acetylcholine release[179,180]. Similarly, in *C. scutulatus scutulatus*, the Mojave toxin is also another PLA₂ complex: one acidic and nonlethal subunit acts as a chaperon for the other basic subunit to improves lethality [44,151,165]. Other examples of synergistic complexes have been found and reported in many species of Viperidae and Crotalidae [76]. Such interactions show the strong synergistic activities in rattlesnake venoms that have been studied intensively through previous endeavors.

Although the synergism referenced previously is studied through the scope of complex formation found in snake venoms, other synergisms were studied through co-administration of major toxins[76]. A prevalent example between major components are SVMP P-III and an acidic PLA₂ in *Bothrops alternatus* called Baltergin and Ba SpII RP4 PLA₂, respectively[181,182]. In which, Baltergin possess high edematogenic and myotoxic activities [181] while PLA₂ has no myotoxicity, although it is the most abundant PLA₂ in this specie [182]. When acting simultaneously, both are able to cause complete detachment of C₂C₁₂ myoblast cells, while none can achieve 50% of detachment on their own[183]. The analogous synergism has also been recorded in endothelial cells, SVMP's natural target[76,184]. The mechanism of synergism for such interaction is said to not based on PLA₂ enzymatic activities but is proposed as through interactions with endothelial cells' membranes, free of catalysis[184]. Furthermore, this PLA₂ does not target the extracellular matrix proteins like SVMP [181], indicating a mechanism 2 of synergism. Both of these enzymes are present in many rattlesnakes' venoms (Table 1 & 3) and their association is also reported through our analysis (Table S1) There are reports indicating the synergism between crotoxin and crotamine, a member of MYO toxins in *Crotalus* venoms, that facilitates the internalization of CB subunit, and increase the neuronal toxicity[76,185]. Unfortunately, these interactions are not found in the analysis (Table 2) although are present in *Crotalus* venoms (Table 1), which could be due to the sparse reports on *Crotalus*' venoms with many species are still under-investigated as stated previously.

There are even fewer studies focusing on synergism between major and minor components: SVSP, a major toxin and BPPs, a minor toxin [76], indicating a biased approach in studying venom toxins produced by current major/minor toxin classification convention. BPPs, which are micromolecular hypotensive peptides in snake venoms, can inhibit angiotensin-converting enzyme and induce hypotensive action of bradykinin, accompanied with hyperpermeability of blood

vessels[68,110,186,187]. Thus, BPPs are targeted for many pharmaceutical developments for treatment of hypertension and heart failure [188,189]. On the other hand, many SVSPs show activities that are similar to kallikrein, a serine proteinases, with the specific and limited proteolytic functions that releases bradykinin [76,146,190]. Previous works indicated that BPPs can act synergistically with kallikrein-like SVSPs, which release bradykinin more effectively than endogenous kallikrein and produce potent hypotension and vascular shock in prey[76,98,146,191-193] (mechanism 1). Such lack of studies regarding minor components shows the flaw in current classification of major and minor venom components, in which the current SVSP-BPP interaction that produces severe physiological consequences is lacking in number of dedicated researches with many aspects of the interaction remain unclear. Likewise, many components with substantial amount of associations with other toxins, like CTL or NGF, are not investigated at all for their potential synergisms. Therefore, there is a need to develop a deeper understanding of minor components in venom of rattlesnakes to discover more associations such as that of SVSP and BPP.

Another way to explain the associations of these toxins is through the evolution of toxins. One relationship that has been explored in previous studies is between SVMP P-III and Dis. Dis are small, non-enzymatic proteins that can bind to extracellular receptors (integrins) with many motifs and sizes, two of which are RGD and MVD motifs[9,71,147,194,195]. While SVMP-PIII is a subclass of SVMP with a Dis-like domain[196,197]. Dis, especially the RGD/MVD motifs, is suggested to be produced from the rapid evolution occurred in the genes coding of SVMP-PIII[194,195,198]. The RGD/MVD motifs of Dis are presented in many *Crotalus* species [194] along with the presence of SVMP-PIII, represent as rule 17 (Table 2) can be explained through this evolution model, although the co-association with LAAO is still largely unknown.

Some associations may not need to be derived through their toxicity but could be explained through the proteins' house-keeping functions. The existence of SVMP inhibitor is thought to be a house-keeping molecule, despite its potential therapeutic activities, which helps neutralize the potent SVMP in the venom glands as a self-defense mechanism[199], yet not many studies have been invested in this family along with the lack of appearance in many rattlesnake venoms, where high amount of SVMP exists (Table 1), indicates a gap in knowledge that requires further efforts. Likewise, NGF is known for its ability to inhibit SVMP proteolysis in Viperidae[200,201]. But growing works have suggested other mechanisms in which NGF can act as cytotoxic proapoptotic factors in tissues that don't have TrkA receptors[200,202,203]; or as ancillary functions, like Hya, to help with efficient absorption of venom component through release of granules molecules (histamine, serotonin, etc.)[178,200,204]. Such large release can also have its own impactful consequences (anaphylaxis, bronchoconstriction, vasodilation, etc.)[200,205]. However, not many *Crotalus* species have NGF as seen previously in Table 1, indicating yet another gap of knowledge in *Crotalus* venomics. Using SVMP as common targeting model to explain the association of NGF and SVMP inhibitor (rule 6, Table 4) is promising, but due to the insufficient amount of information provided, such explanation warrants further attempts in co-administration testing to confirm.

4. Conclusions

Through this meta-analysis we have discovered myriad of associations many of which are yet to be described, but they do provide promising potential synergistic effects that are worth investigating. For example, using rules 2 and 13 in *Crotalus* venoms (Table 2), CTL, a

protein/glycoprotein that specifically binds to carbohydrate moieties and glycoconjugate, can target and interact with platelet receptors and blood coagulation factors[206], which are also targets for Dis[207], indicate their potential synergisms with antiplatelet toxins and assert the hypotensive results along with many other toxin groups like SVSP[76,98,146,191-193]. Thus, there is a need to characterize toxin components and their associations within the toxicology studies as well as for pharmaceutical interests[72]. With an increase amount of characterization studies, novel families may also be correctly added into the venom profiles such as three-finger toxins (3FTx), which often is present in elapids and a few occasions in rattlesnakes' genome and transcriptome [14-16,168,170]. Furthermore, similar studies for *Sistrurus* species are still less abundant than their relative *Crotalus* species, indicating an underdeveloped field of study that warrant further interests. Additionally, this work also address the problem of conventional classification of venom toxins as major or minor based on importance and ubiquity, which are often MYO, PLA₂, SVMP and SVSP [13,131], as the cause of much more attention on these dominant toxin families and overlooking other protein families such as Growth Factors. Therefore, we highlight the importance of studying venom components not only as individual components but also in understanding the relationship between them. It is by the combination of ubiquity, bioactivity, and relationship between the protein families that we can classify the venom components as major or minor.

5. Materials and Methods

We collected articles and abstracts on venom for each *Crotalus* and *Sistrurus* species through the following: databases (PubMed, ScienceDirect, Scopus, Google Scholar, Web of Science), journal's databases (BMC Genomics, Journal of Proteome Research, Journal of Proteomics, Toxicon, Toxicology, Toxins), publisher databases (Wiley Online Library, MDPI, Elsevier). We used "venom" OR "proteomic" OR "venomic" OR "transcriptome" OR "proteome" AND "Name of the species" as keywords for conducting our search. We also, examined references in studies produced from the search results for any additional information. Collected records were earliest obtainable records to those that are published in January of 2020.

From collected records, any article that did not contain information regarding venom composition and components of any *Crotalus* and *Sistrurus* species was not used in the current analysis. Otherwise, the articles' full-text version would be further assessed with the following inclusion and exclusion criteria.

For the article to be included in the current analysis, it had to fulfill one of the following criteria: (1) report proteome or transcriptome profile of the venom of any corresponding species; (2) report at least 1 toxin family/component, which is not artificially synthesized based on another similar toxin component; (3) be a comparative study reporting transcriptome/proteome profile for *Crotalus*, *Sistrurus* species/subspecies; (4) studies that report variability in venom components for any *Crotalus*, *Sistrurus* species/subspecies.

The following criteria were used to exclude any study from the current analysis: (1) reviews that focuses on toxin families and/or articles focuses on the genomic evolution of toxin families; (2) articles with no transcriptome/proteome profiles; (3) articles with no data on toxin family isolated from venom; (4) articles that focuses on new artificially synthesized molecules, based on similar toxin component or recombinant protein/peptides in venom; (5) articles reporting methods to inactivate toxin family from rattlesnakes; (6) case study on rattlesnakes' bites; (7) studies describing methods to

detect toxin families/components. From the studies that fulfilled our inclusion criteria and did not meet any exclusion criteria we collected and compiled all venom constituents that are reported for each species in the genus *Crotalus* and *Sistrurus* in Table 1 and Table 3, respectively. The compiled data was cross-checked by authors for correctness and confirmations.

Using the data from Table 1 and 3 we performed two separate Frequent Item-set data mining analysis for *Crotalus* and *Sistrurus* venoms. To conduct Frequent item-set data mining we used only presence absence data. Frequent item-set data mining helps in identifying the association rules associated with the expression of different proteins in venom. Studies on *Sistrurus* venom components are sparse, thus, can introduce bias towards data-mining analysis. The rules specify the confidence, lift, and support for specific proteins to occur together in venom. Support is defined as absolute frequency, i.e., a support of 25% means that venom components x, y, and z will occur together in 25 % of all venom. Confidence is correlative frequency. i.e., a confidence of 60% means that if x and y occur, then 60% of times z will also occur. An association rule is valid only if lift is greater than 1. Higher the value of the lift, the higher is the validity of the rule. Since, many studies associated with rattlesnake venom concentrated on highly abundant species or species containing more “major components”, this affects the performance of the statistical models due to presence of null values. Since, we did only used presence absence data for toxin families from individual studies the chances of bias from individual studies affecting our results were low. With increase in venom composition and variation data, the associations produced by Frequent Item-set data mining analysis will be more informative. All analysis was performed using the software R (R Core Team, 2019).

Supplementary Materials:

The following is available in the supplementary material attachment. Table S1: Depictions of full association rules between proteins expressed in *Crotalus* venom.

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Abbreviations

The following abbreviations are used in this manuscript:

Abbreviation	Full Name
3FTx	Three-finger toxin

5'-NT	5'-nucleotidase
Achase	Acetylcholinesterase
ANP	Natriuretic peptide type A
ATPase	Adenosine triphosphatase
BIP	Bradykinin inhibitory peptide
BNP	Natriuretic peptide type B
BPP	Bradykinin potentiate peptide
CTL	C-type Lectins
CNP	Natriuretic peptide type C
CysProt	Cysteine Protease
CRiSP	Cysteine-rich secretory protein
CA	crotapotin
CRTX	Crotoxin
Dis	Disintegrin
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GC	Guanylyl cyclase
Hya	Hyaluronidase
Kazal	Kazal-type inhibitor
Kun	Kunitz-type inhibitor
LAAO	L-amino acid oxidase
MTX	Mojave toxin

MYO	Myotoxin
NGF	Nerve growth factor
OHA	Ohanin
PDE	Phosphodiesterase
PDGF	Platelet derive growth factor
PLA ₂	Phospholipase A ₂
PLB	Phospholipase B
PLD	Phospholipase D
SVMP	Snake venom metalloprotease
SVSP	Snake venom serine protease
TLE	Thrombin-like enzyme
VEGF	Vascular endothelial growth factor
WAP	Waparin

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