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Transcriptome analysis of the Amazonian viper *Bothrops atrox* venom gland using expressed sequence tags (ESTs)

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ABSTRACT

Bothrops atrox is a highly dangerous pit viper in the Brazilian Amazon region. We produced a global catalogue of gene transcripts to identify the main toxin and other protein families present in the *B. atrox* venom gland. We prepared a directional cDNA library, from which a set of 610 high quality expressed sequence tags (ESTs) were generated by bioinformatics processing. Our data indicated a predominance of transcripts encoding mainly metalloproteinases (59% of the toxins). The expression pattern of the *B. atrox* venom was similar to *Bothrops insularis*, *Bothrops jararaca* and *Bothrops jararacussu* in terms of toxin type, although some differences were observed. *B. atrox* showed a higher amount of the PIII class of metalloproteinases which correlates well with the observed intense hemorrhagic action of its toxin. Also, the PLA_2 content was the second highest in this sample compared to the other three *Bothrops* transcriptomes. To our knowledge, this work is the first transcriptome analysis of an Amazonian rain forest pit viper and it will contribute to the body of knowledge regarding the gene diversity of the venom gland of members of the *Bothrops* genus. Moreover, our results can be used for future studies with other snake species from the Amazon region to investigate differences in gene patterns or phylogenetic relationships.

1. Introduction

The genus *Bothrops* (Viperidae) comprises more than 30 species and subspecies of snake that inhabit distinct geographical regions of Central and South America (Cidade et al., 2006). In the Brazilian Amazon, *Bothrops atrox* (commonly known as the Amazonian lancehead) is the pit viper most often involved in human snakebites. López-Lozano et al. (2002) reported that more than 90% of snakebite patients who sought medical care at the Medicine Tropical Institute (IMT – Manaus-AM) were victims of this species of pit viper. Available statistics from Amazonia are underestimated in general because many victims fail to reach a hospital before dying.

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The principal clinical effects of poisoning by *B. atrox* are life-threatening bleeding caused by blood coagulation disturbance, shock, and renal failure. Necrosis and bacterial infection at the site of the bite may result in permanent physical disabilities.

In Brazil, the treatment for envenonmation by any *Bothrops* species involves the use of polyspecific *Bothrops* antivenoms. The absence of *B. atrox* venom in the preparation of this antivenom suggests that its efficacy against this venom results from the extensive cross-reactivity of *Bothrops* antigenic epitopes. Nevertheless, in the northern regions of Brazil, snakebite treatment utilizing bothropic antivenom has less effect on envenomed patients largely due to the lack of neutralization of the myotoxic effect. Although clinical studies have demonstrated the efficacy of polyspecific antivenom (Otero-Patiño et al., 1998; Pardal et al., 1994), it is important to know how the variability existent among snake venoms can affect the antivenom response.

Variation in snake venoms has been described not only among species but also within a species. These variations are caused mainly by ontogenetic changes that affect the biochemical and pharmacological profiles of the venom (Saldarriaga et al., 2003). The genus *Bothrops* is a heterogenous group of snakes that display wide variations in their venom's electrophoretic patterns (Chippaux et al., 1991). Geographical variations in venom protein profiles of *Bothrops asper* venom have been observed by Alape-Giron et al. (2008). Additionally, López-Lozano et al. (2002) and Guércio et al. (2006) observed differences in metalloproteinases and toxin content in *B. atrox* venom. Salazar et al. (2007) have also demonstrated several differences among *B. atrox* venoms collected from Bolivia and Brazil. Sexual dimorphism has also been shown to play a role in determining the different venom compositions of *Bothrops jararaca* (Furtado et al., 2006).

There are very few studies that examine the composition of *B. atrox* venom and currently there is any protocol for standardization of this venom. Furthermore, there are very few protein sequences available concerning to *B. atrox* venom. Using a proteomic approach, Guércio et al. (2006) identified the main toxin classes in venom of juvenile, subadult and adult members of the *B. atrox* species. They found that proteins in these venoms belong mainly to the classes of metalloproteinases, lectins, serino-proteinases, bradykinin-potentiating peptides (BPPs) and phospholipases A2.

In the present work, we investigate the pattern of toxin expression in the *B. atrox* venom, based on the analysis of expressed sequence tags (ESTs) from the venom gland cDNA library of a single sub-adult specimen.

Such an approach was previously conducted to describe the venom transcriptomes of *Bothrops insularis* (Junqueira-de-Azevedo and Ho, 2002), *Bothrops jararacussu* (Kashima et al., 2004) and *B. jararaca* (Cidade et al., 2006). In addition to providing new information about toxin transcripts, such studies revealed significant similarities in the patterns of gene expression in the venom gland of these three species of *Bothrops*.

In the present report, a global survey of *B. atrox* ESTs will provide information that can be compared to existing *Bothrops* transcriptome datasets, and can offer supporting evidence for published proteomic data. The results of this survey can also serve as the basis for a molecular phylogenetic study of toxin evolution.

2. Material and methods

2.1. cDNA library construction

A young male adult specimen of *B. atrox* captured in the Manaus region (Amazonas State, Brazil) and maintained in the Herpetarium of the Tropical Medicine Institute – IMT-AM, Manaus was kindly provided by Dr. Jorge Luiz López-Lozano's research group.

Snake venom was extracted by milking. Seventy-two hours after milking, the animal was anesthetized with CO_2 and sacrificed by decapitation. Then, the pair of venom glands was dissected and kept in liquid nitrogen until use.

Total RNA was prepared from the venom glands using the Trizol reagent (Invitrogen Life technologies, CA, USA). The RNA quality and quantity were verified by examining a denaturing agarose electrophoretic gel and then spectrophotometrically using the ratio of absorbances at 260 and 280 nm. A directional *B. atrox* venom gland cDNA library was constructed using the Creator Smart cDNA Synthesis System (Clontech, Mountain View, CA, USA). First strand of cDNA was synthesized from total RNA and the second strand was obtained by long distance PCR (LD-PCR), following the manufacturer's instructions.

Small sized and truncated cDNAs were removed by size fractionation of the entire library through a CHROMA SPIN-400 column. We collected 13 fractions of which the first five and last three have been discarded. The five remaining fractions were pooled and an aliquot of each was ligated into a pDNR-Lib vector. *Escherichia coli* TOP10 (Invitrogen Life Technologies, CA, USA) cells were transformed with recombinant plasmids by electroporation and spread onto LB plates that contained chloramphenicol (34 μg/mL). To verify the quality of the library, bacterial clones were randomly chosen and the recombinant plasmid was rescued. Then, the plasmid was digested with Eco RI and HindIII to check the insert sizes.

A set of 1056 independent clones were selected and inoculated into 96 well microplates with CircleGrow broth (containing chloramphenicol), and then cultured for 22 h. Plasmids were prepared using the alkaline lysis method (Sambrook et al., 1989). This was followed by filtration on a MultiScreen MAGVN250 (Millipore), precipitation with 70% ethanol and resuspension in sterile deionized water (Vasconcelos et al., 2003).

The plasmids were sequenced using the chain termination method (Sanger et al., 1977) on a DYEnamic ET Terminator Kit (GE Healthcare, Fairfield, CT, USA) and then they were analyzed on a MegaBace 1000 automated sequencer (GE Healthcare, Fairfield, CT, USA).

2.2. Assembly and identification of ESTs

EST electropherogram files were exported for automated analysis by the TIMINA (http://timina.sourceforge.net/wiki/) pipeline for sequence analysis and clustering (Brígido et al., 2005). Sequence quality was first analyzed

with the Phred and Crossmatch software packages to remove low quality ends (Green, 1996). After this preliminary analysis, only good quality sequences (phred > 20) with a length longer than 150 bp were displayed in the table for definitive annotation.

The collection of good quality sequences was organized into clusters with CAP3 software. We took into account overlaps of 50 bp that had at least 98% identity (Huang and Madan, 1999). The clustered sequences were compared to protein GenBank NR (http://www.ncbi.nih.gov) and Swissprot release 44 (ftp.ebi.ac.uk/pub/databases/swissprot/release/) databases using the BLASTx program (Altschul et al., 1997). Gene descriptions and EC numbers from Swissprot best hits and their associated product names were automatically assigned using 10^{-10} as the *e*-value cut off. Thereafter, the ESTs were manually inspected by comparing the BLAST results with the automatically annotated EC numbers for functional classification. After this an additional annotation allowing the identification of protein domain architectures was conducted comparing the clusters sequences with the Swissprot, SP-TrEMBL and stable Ensembl proteomes databases using the SMART software (Schultz et al., 2000).

Finally an alignment was done with the sequence of best hit for each cluster using MegAlign software (Lasergene, Madison, WI, USA).

3. Results and discussion

3.1. Analysis of cDNA quality and sequence clustering

The cDNAs lengths were confirmed by agarose gel electrophoresis of digests of randomly chosen clones. Results showed that synthesized cDNAs encompassed a range of 200–2000 bp long.

The sequencing procedure rendered a total of 610 high quality sequences of which 402 were clustered in 66 contigs. By querying the NCBI databases with *B. atrox* ESTs, we identified 211 ESTs that matched snake venom toxins (59.4%). These ESTs formed 26 clusters of which BATC21, 30, 48, 65 and 163 represent full sequences of known protein domains.

This was rather different from what has been observed in *B. jararaca* (77.6%) (Cidade et al., 2006), *B. jararacussu* (71.6%) (Kashima et al., 2004) and *B. insularis* (67%) (Junqueira-de-Azevedo and Ho, 2002).

Aside from the toxin transcripts, we found 146 ESTs matching proteins involved in cellular processes (41.1%), 154 ESTs with no database matches (25.2%) (these might represent unique proteins in the venom), and 99 ESTs matching described proteins with no functional attributes (16.2%) (Table 1).

All obtained clusters were deposited in the EST database of GenBank (http://www.ncbi.nlm.nih.gov/dbEST) under accession numbers FL591281–FL591890.

3.2. ESTs identification and predictive functional assignment

The ESTs of *B. atrox* were grouped in transcripts coding for proteins related to cellular processes and toxins (Table 1).

Table 1
Representation of ESTs in the *B. atrox* venom gland.

Function	No. of ESTs	% of total	% of matching
Similar to toxins	211	34.6	59.4
Similar to cellular processes proteins	146	23.9	41.1
No database match	154	25.2	–
Unknown proteins	99	16.2	–

Comparing the proportion of *B. atrox* transcripts encoding toxins to that proportion in other transcriptomes, there is an abundance of such transcripts preferentially expressed in *Bothrops* venom glands (Fig. 2) (Cidade et al., 2006; Kashima et al., 2004; Junqueira-de-Azevedo and Ho, 2002).

In *B. atrox*, it was also observed that the major classes of toxins are also found in *B. jararaca*, *B. jararacussu* and *B. insularis* (Table 2). The cDNAs coding for the main toxins found in the *B. atrox* venom gland are listed in Table 3.

3.2.1. Metalloproteinases/desintegrins

As observed in *B. insularis* (Junqueira-de-Azevedo and Ho, 2002) and in *B. jararaca* (Cidade et al., 2006), toxin transcripts for metalloproteinase are also the most abundant toxin transcripts in *B. atrox* venom gland (61.6% of total toxin ESTs). Moreover, this percentage is the highest among the four *Bothrops* species (Figs. 1 and 2).

According to current classifications, snake venom metalloproteinases (SVMPs) are usually divided into four classes depending on the organization of their domains (Hite et al., 1994). These classes are PI (metalloproteinase domain), PII (metalloproteinase and desintegrin domains), PIII (metalloproteinase, desintegrin and cysteine-rich domains) and PIV (same PIII domains plus a lectin-like domain linked by disulfide bonds). Recently Fox and Serrano (2005) proposed a classification which divided PII and PIII classes into subclasses reflecting the potential for proteolytic processing and the formation of dimeric structures.

SVMPs also known as hemorrhagins are associated to diverse functional activities interfering with the blood coagulation and hemostatic plug formation or by degrading the basement membrane or extracellular matrix components (Matsui et al., 2000) and other more recent studies

Table 2
Putative toxin transcripts classes identified in *B. atrox* venom gland transcriptome.

Toxins	No. of clusters	No. of ESTs	% of total	Redundancy
Metalloproteinase	11	130	21.3	11.8
Serine-proteinase	4	17	2.8	4.3
C-type lectin	5	14	2.3	2.8
Phospholipase A_2	1	28	4.6	28
BPP*	1	15	2.5	15
LAO	1	3	0.5	3.0
svVEGF	1	2	0.3	2.0
Cysteine-rich protein	1	1	0.2	1.0
PLA_2 inhibitor	1	1	0.2	1.0
Total	26	211	34.6	

BPP – Bradykinin-potentiating peptide; svVEGF – snake venom vascular endothelium growth factor; LAO – L-amino acid oxidase.

Table 3
Cluster identification and putative toxin-encoding clusters in *B. atrox* venom.

Cluster	Reads	Annotation	*e*-value
Metalloproteinases			
BATC01	19	Bothrostatin precursor [*Bothrops jararaca*]	1.0E−104
BATC16	72	Jararhagin precursor [*Bothrops jararaca*]	0.0
BATC19	4	HR1a metalloproteinase [*Trimeresurus flavoviridis*]	2.0E−58
BATC41	2	Halysase [*Gloydius halys*]	1.0E−35
BATC44	9	Metalloprotease BOJUMET II [*Bothrops jararacussu*]	1.0E−110
BATC47	2	Vascular apoptosis-inducing protein [*Trimeresurus flavoviridis*]	1.0E−71
BATC61	3	Hemorrhagic metalloproteinase HF3 [*Bothrops jararaca*]	5.0E−47
BATC62	16	Desintegrin Jararacin precursor [*Bothrops jararaca*]	2.0E−48
BATC187	1	Hemorrhagic metalloproteinase HF3 [*Bothrops jararaca*]	1.0E−120
BATC213	1	Metalloproteinase precursor [*Bothrops insularis*]	9.0E−82
BATC255	1	VLAIP-B [*Macrovipera lebetina*]	2.0E−89
Serine proteinases			
BATC20	10	Venom serine proteinase HS112 precursor [*Bothrops jararaca*]	1.0E−93
BATC58	2	Venom serine protease BthaTL [*Bothrops alternatus*]	
BATC66	4	Venom serine proteinase HS114 precursor [*Bothrops jararaca*]	
BATC149	1	Venom thrombin-like enzyme [*Deinagkistrodon acutus*] (acubin)	7.0E−20
C-type Lectins			
BATC30	2	Bothrojaracin chain A precursor [*Bothrops jararaca*]	2.0E−37
BATC48	5	Botrocetin B chain [*Bothrops jararaca*]	1.0E−102
BATC65	5	C-type lectin [*Bothrops insularis*]	1.0E−76
BATC163	1	Stejaggregin-A alpha chain [*Trimeresurus stejnegeri*]	1.0E−69
BATC180	1	Renin biding protein C-type lectin like	
Phospholipase A2			
BATC21	28	Hypotensive Phospholipase A2 [*Bothrops jararacussu*]	7.0E−55
Phospholipase A2 inhibitor			
BATC178	1	Phospholipase A2 inhibitory protein Annexin A1	2.0E−28
Cysteine-rich venom proteins			
BATC145	1	Ablomin [*Agkistrodon blomhoffi*]	1.0E−108
Bradykinin-potentiating peptide precursors			
BATC51	16	mRNA for Bradykinin-potentiating peptide [*Bothrops* jararacussu]	0.0
Others			
BATC08	2	Vascular endothelial growth factor [*Trimeresurus flavoviridis*]	1.0E−28
BATC13	3	L-Amino acid oxidase precursor [*Bothrops jararacussu*]	1.0E−115

indicated that these proteins have another functionalities as apoptotic and inflammatory activities (Fox and Serrano, 2005).

B. atrox transcriptome clusters showed a high redundancy of PIII metalloproteinase homologs, a single assembling group was identified as a PI metalloproteinase and another single assembling group assigned as a PII metalloproteinase homolog. The BATC16 cluster contains most of the metalloproteinase transcripts (55.38%), and shares similarity with the *B. jararaca* metalloproteinase jararhagin (Paine et al., 1992). This cluster had a high similarity score in sequence with jararhagin metalloproteinase and desintegrin domains (85.2%) as well an identical zinc metal biding site and DECD.

Three clusters, BATC41, BATC47 and BATC255, are similar to the apoptosis-inducing proteins. BATC41 had 89.7% of similarity with ACR (Adam Cysteine-Rich domain) of halysase from *Gloydius halys* (You et al., 2003), BATC47 had similarity with desintegrin and ACR domains of VAIP from *Trimeresurus flavoviridis* (Masuda et al., 2001) with 80% of identity, and BATC255 was 56.7% similar with M12B propeptide and reprolysin domain of VLAIP-B from *Macrovipera lebetina* (Trummal et al., 2005). These proteins hydrolyze the α-chain and (more slowly) the β-chain of fibrinogen.

The BATC44 cluster has a high similarity with metalloproteinase *B. jararacussu* BOJUMET II (Kashima et al., 2004). It possesses the identical desintegrin domain SECD and a high identity score (89%) with desintegrin and ACR domains. Two other clusters, BATC61 and BATC187, have been assigned as *B. jararaca* HF3 homologs. In the first cluster, sequence similarity is limited to an ADAM cysteine-rich domain with 81% similarity. In the second cluster, we identified both desintegrin and cysteine-rich domains that reach a similarity of 76.3% to the apoptosis-inducing protease homolog from the viper *M. lebetina*. The cluster BATC62 was identified as a *B. jararaca* jararacin homolog and was identified as a reprolysin domain. Finally, the cluster BATC213 showed similarity with the reprolysin family domain (87.2%) and it was assigned as a *B. insularis* metalloproteinase precursor homolog. BLAST searches for the BATC19 cluster showed that it shared 63.4% of similarity with ACR domain of a *T. flavoviridis* HR1a metalloproteinase (Kishimoto and Takahashi, 2002).

A single cluster was identified as a PII metalloproteinase homolog. BATC01 was the second most represented group (19 sequences) representing 14.6% of the total toxin transcripts. BLAST results showed 82% of similarity between it and the *B. jararaca* bothrostatin reprolysin family propeptide domain (Fernandez et al., 2005).

An analysis of all metalloproteinase clusters showed a similar pattern to that observed in *B. jararaca* and *B. insularis* concerning PII and PIII putative homolog identification. PIV proteins or precursors were not found in our EST dataset.

One interesting observation surrounds the predominance of transcripts encoded metalloproteinase PIII class in *B. atrox* venom gland just like in the *B. insularis* transcriptome (Junqueira-de-Azevedo and Ho, 2002). Since

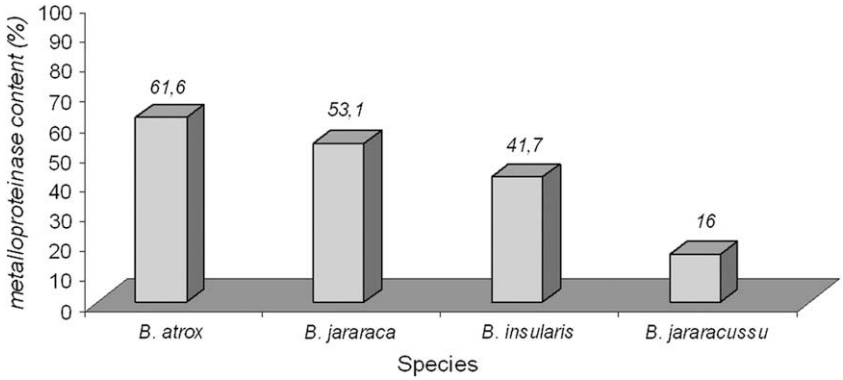

Fig. 1. Graphics showing the comparison of metalloproteinase transcripts in the venom gland of four *Bothrops* species.

members of PIII class cause inhibition of collagen-induced platelet aggregation through their interactions with integrin α2β1 (Kamiguti et al., 2003; Jia et al., 2000) (this activity may even be enhanced by the cysteine-rich domains these proteins), they are considered as one of the most potent hemorrhagins (Gutiérrez et al., 2005; Markland, 1998). Manual annotation using SMART allowed predicting metalloproteinase domains for clusters BATC01, BATC16, BATC62, BATC213 and BATC255. For the clusters BATC19, BATC41 and BATC61 it was predicted only ACR domain and for the clusters BATC44 and BATC47 it was identified both desintegrin and ACR domains characteristic of PIII metalloproteinases.

It is important to consider that posttranslational modifications are common in this class of proteins. PII metalloproteinases transcripts can be processed giving rise to PI metalloproteinases (Takeya et al., 1992) and PIII metalloproteinases to short desintegrins or PIV metalloproteinases. Thus the predominance of cDNAs encoding PIII metalloproteinases not necessarily means a high concentration of protein in the venom.

Besides the presence of certain domains does not necessarily denote hemorrhagic capability although in terms of potency (Fox and Serrano, 2005).

Nevertheless clinical data show the hemorrhagic character of *B. atrox* venom and our results may provide support to reinforce and helps us understand how it could be as toxic as *B. insularis* venom and more potent than *B. jararaca* venom (Cidade et al., 2006).

3.2.2. Phospholipase A_2

Phospholipases A_2 (PLA $_2$) are enzymes which hydrolysing specifically the acid–ester linkage in the sn-2 position of glycerophospholipids releasing fatty acids from lysophospholipids. These enzymes have high toxicity and it is believed that venom PLA_2s evolved from pancreatic enzymes that aid in the digestion of prey by gene duplication and changes in regions of exons (Kini and Chan, 1999) acquiring a wide range of pharmacological effects such neurotoxicity, coagulation and anticoagulation, myotoxicity, edema induction and cardiotoxicity (Dunn and Broady, 2001).

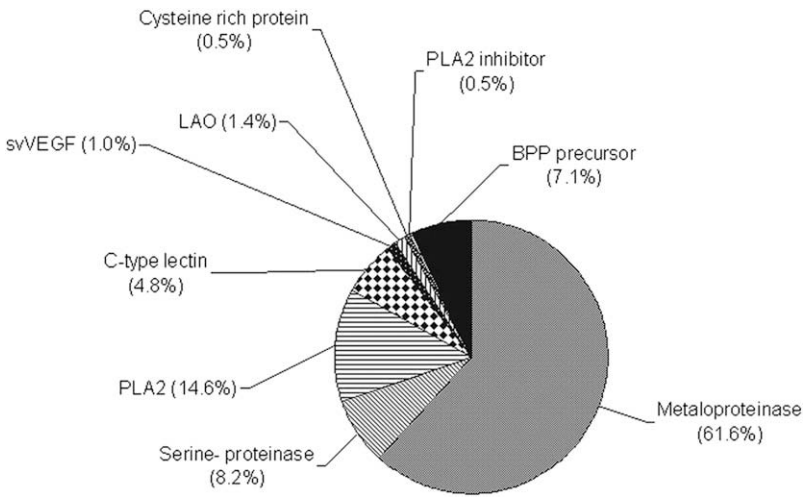

Fig. 2. Graphics showing the EST toxin transcripts content in *B. atrox* venom gland. BPP – Bradykinin-Potentiating Peptide; PLA2 – phospholipase A2; svVEGF – snake venom vascular endothelium growth factor; LAO – L-amino acid oxidase.

PLA_2 is largely responsible for the intense local pain and damage caused by *Bothrops* bites. An inflammatory response is initiated through the enhancement of vascular permeability and neutrophil recruitment (Zuliani et al., 2005). The cDNAs coding for these classes of enzymes were the second most frequently observed in the *B. atrox* transcriptome, although they showed a high redundancy in only one cluster. This cluster, named BATC21, is comprised of 28 ESTs sharing 76.8% similarity with the *B. jararacussu* hypotensive PLA_2 homolog (Andrião-Escarso et al., 2000). Cluster inspection showed conserved His48, Asp49, Tyr52 and Asp99 key residues at the catalytic site. In addition the residues comprising the calcium-biding loop Tyr28, Gly30 and Gly32 were identified as is the case with *B. jararacussu* PLA_2.

3.2.3. Serine proteinases

Serine proteinases affect hemostasis by acting upon coagulation cascade components. These enzymes act predominantly as anticoagulants due to their consumption of coagulation factors (Markland, 1998). The common catalytic amino acids for these enzymes are serine, histidine and aspartate.

In our database, we found that four clusters could be assigned as serine proteinase homologs. BATC20 and BATC58 were assigned as VSP HS112 precursors of *B. jararaca* and VSP BthaTL of *Bothrops alternatus* homologs and the similarity scores were 82% and 84.1% respectively. Nevertheless, both clusters also showed a slight similarity (70%) to batroxobin – a thrombin-like enzyme isolated from *B. atrox* venom that releases fibrinopeptide A from fibrinogen (Itoh et al., 1987). BATC66 and BATC149 clusters were exclusively identified as thrombin-like enzyme homologs isolated from *B. jararaca* (VSP HS114) with 85.5 % of similarity (Saguchi et al., 2005) and *Deinagkistrodon acutus* venoms (Zha et al., 2006) with similarity score of only 23%.

Though the three of four clusters showed high similarity with thrombin-like enzymes, none seemed to be present in the complete catalytic triad, HDS. The BATC20 and BATC66 clusters, for example, lack the serine residue. In the BATC58 cluster, a replacement of the Hys residue with Asp residue was verified and this substitution probably causes an abolishment of catalytic activity. A complete lack of the typical catalytic residues was found in BATC149; therefore it is likely that BATC58 and BATC149 clusters do not represent functional serine proteinases.

3.2.4. C-type lectins

The C-type lectin-like proteins are enzymes that have also been shown to act on various parts of the coagulation cascade. They exhibit anticoagulant and procoagulant effects depending on their biding characteristics. They can inhibit the conversion of prothrombin into thrombin by binding to coagulation factors IX and X (Lee et al., 2003) or they can directly inhibit thrombin by binding to its exosites 1 and 2 (Arocas et al., 1997). They can also inhibit platelet aggregation by binding to GPIb (Shin et al., 2000) and stimulate platelet aggregation through activation of the Willebrand factor.

Amino acid sequences of CLP are commonly almost identical (15–40%) to the carbohydrate-recognition domain (CRD) of C-type lectins including mammalian mannose-binding protein (MBP) (Weis et al., 1991, 1992) and snake venom galactose-specific lectin (Hirabayashi et al., 1991).

The *B. atrox* transcriptome showed four clusters assigned as putative distinct members of the C-type lectin family. All cluster had similarity with CRD (carbohydrate-recognition domain) typical of this proteins. BATC30 was 60% identical to bothrojaracin chain A precursor (Arocas et al., 1997) which directly binds thrombin. It is interesting to note that in this cluster a region prior to CRD domain was identified as a transmembrane domain by SMART program. This region was very similar to signal peptide of bothrojaracin with two residues substitutions (Lys5 – Ile5; Val13 – Phe13). BATC48, with 71% similarity to bothrocetin (Usami et al., 1993) could bind to and activate the von Willebrand factor and consequently its interaction with platelet glycoprotein (GP) Ib (Sen et al., 2001). BATC65 is 91.9% identical to *B. insularis* C-type lectin and might present hemagglutinating activity. BATC163 is 74.7% similar to Stejaggregin-A obtained from *Trimeresurus stejnegeri* venom and is another cluster of C-type lectin.

3.2.5. Bradykinin-potentiating peptides (BPPs)

BPPs (Bradykinin-potentiating peptides) are proline-rich peptides formed from 5 to 14 amino acids with a pyroglutamyl residue at the N-terminus and an Ile-Pro-Pro (IPP) sequence at the C-terminus. These peptides act by inhibiting the angiotensin-converting enzyme (ACE) preventing the formation of angiotensin II from angiotensin I (Hayashi and Camargo, 2005) and thus acting as vasodilator.

BPPs are involved with the hypotensive effect of the venom of the *Bothrops* species. Because of this, they have been used as models for the development of new antihypertensive drugs (Fernandez et al., 2004).

Surprisingly, we were not able to find any clusters for these peptides. However, using BLASTn we were able to identify a single cluster (BATC51 with 15 ESTs) that matched the long 5′ UTR of a BPP mRNA from *B. jararacussu*.

3.2.6. Others toxins

In the *B. atrox* EST database, we found another cluster coding for toxins such cysteine-rich secretory proteins (CRISPs), L-amino acid oxidase (LAO) and snake venom vascular endothelium growth factor (svVEGF).

In the literature, little information about the action of CRISPs is known, although they seems to inhibit the smooth muscle contraction and block the triggering of the cyclic-nucleotide-gated ion channels (Yamazaki and Morita, 2004; Oispov et al., 2005). On the other hand, LAO induces apoptosis and creates indirect hemorrhagic effects by affecting platelet function. Snake venom (sv-)VEGFs act by increasing vascular permeability which probably contributes to the hypotensive action of the venom by facilitating the dispersion of its components (Junqueira-de-Azevedo et al., 2001).

3.3. Clusters related to cellular functions

Clusters encoding proteins with common cellular functions comprise 41.1% of the *B. atrox* venom gland transcripts. The clusters were categorized in groups according

to the biochemical pathways that their corresponding proteins are presumed to be involved in. Transcripts related to general metabolism were the most abundant in our database followed by transcription and translation related functions (Fig. 3). However, this was not the same as the pattern of expression observed in *B. insularis* (Junqueira-de-Azevedo and Ho, 2002) and *B. jararaca* (Cidade et al., 2006). Most transcripts found in this last category are for ribosomal proteins and elongation factors. According to Junqueira-de-Azevedo and Ho (2002), this result might be explained by what is expected for a specialized tissue in which the main function is to produce and secrete toxic proteins into the lumen of venom glands.

Among transcripts for protein processing, our database reveals clusters for calnexin, calmodulin, and other EF hand protein members. All of these belonging to the calcium binding protein family. Calmodulins are exclusively expressed in the venom gland from many species, but they are neither secreted into the venom nor contribute to the makeup of the venom. Their function is related to the export of toxins out of the cell and they may play a fundamental role in toxin secretion (Junqueira-de-Azevedo and Ho, 2002). Calnexin belongs to the ER chaperone system, which is responsible for the correct folding of proteins. Two clusters encoding protein disulfide isomerase (PDI) were also found. These are redox proteins responsible for disulfide bond assembly (Freedman et al., 1994).

We identified five clusters for reverse transcriptase. Three of them were from the teleost LINE family (Duvernell and Turner, 1998) and one was a transposase. We cannot identify any transcript related to retrotransposable elements previously identified in *B. insularis* (Junqueira-de-Azevedo and Ho, 2002), but the presence of reverse transcriptase and transposase transcripts suggests the presence of retro-elements in the cells of *B. atrox* venom glands. Another uncommon occurrence in the *B. atrox* transcriptome was the detection of two ESTs encoding cytosolic phospholipase A2 and phospholipase B-like proteins. These proteins had not previously been described in snake venoms. Therefore, we reasoned that we should keep these clusters in the group of ESTs related to cellular processes for further investigation.

Additionally, we found an EST encoding for the protein annexin, which is also known as lipocortin. Cluster BATC178 had a similarity score of 36.1% with Annexin A1 from *Sus scrofa*. This protein binds to phospholipids in a calcium-dependent manner and a complex set of functions was reported for these proteins, including inhibition of phospholipase A_2 activity (Seemann et al., 1996). The annexin homolog was identified for the first time as a venom gland transcript in *B. jararaca* (Cidade et al., 2006) and, until now, that had been the only known occurrence of this homolog.

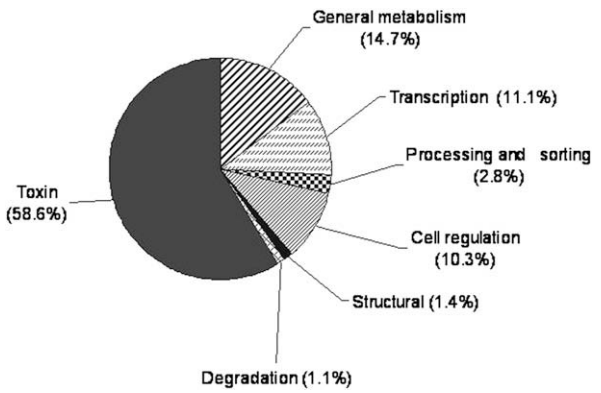

Fig. 3. Graphics showing toxin and cellular processes transcript relationship in the *B. atrox* venom gland.

Alignment of BATC178 sequence with 31 $sPLA_2$ and one pancreatic cPLA2 sequences allowed concluding that this is not a specific region of low molecular weight phospholipase A2 capable of inhibiting its activity (Cordella-Miele et al., 1993). Thus further studies are needed to verify whether this cDNA really encoding an annexin PLA_2 inhibitor.

4. Discussion

Comparing our dataset with those of the other three *Bothrops* transcriptomes (Junqueira-de-Azevedo and Ho, 2002; Kashima et al., 2004; Cidade et al., 2006), we noticed some similarities and principally some noteworthy differences. The major classes of transcripts expressed in *B. atrox* venom gland are those belonging to a class of metalloproteinases as is the case in *B. insularis* and *B. jararaca*. Nevertheless, this family of enzymes in *B. atrox* transcriptome represents more than 60% of all ESTs. Such number is the highest among the four *Bothrops* transcriptomes known.

Another interesting fact is the high content of PIII metalloproteinases transcripts, which is also similar to the *B. insularis* venom gland. PIII metalloproteinases are the most potent toxins from this family and their predominance in *B. insularis* venom contributes to its higher toxic effects compared to *B. jararaca* venom (Melgarejo, 2003). This deserves attention because *B. atrox* and *B. insularis* inhabit greatly different ecosystems and geographical regions that are very distant, respectively, the rain forest and a South Atlantic island. Our results surprisingly show a similar pattern of toxin expression in the venoms of both species.

It is known that the composition of snake venom depends on diverse ontogenetic and epigenetic factors such as like geographic distribution, diet, age and sex (Chippaux et al., 1991; Daltry et al., 1996). Moreover, endemism seems to influence snake venom of snakes inhabiting islands. This is the case for *B. insularis*, in which its venom evolved according to the presence of high snake density and low food availability in Queimada Grande Island (São Paulo coast, Brazil).

On the other hand, *B. atrox* lives in a habitat in the rain forest, where great quantities and large diversity of prey (food) are available. There are also many ontogenetic factors that may operate on venom evolution. Young *Bothrops* snakes prey on lizards, birds and amphibians and preferentially start eating small mammals when they become adults (Daltry et al., 1996).

In fact the relative number of toxin sequences belonging to a given protein family may not correlate with the relative abundance of this toxin family in the venom, due

posttranslational modifications which frequently occurs. Nevertheless our results corroborate a proteomic study in which are evidenced the qualitative and quantitative changes in the toxins content of juvenile, sub-adult and adult crude venom of *B. atrox* from Amazonian rain forest recently published (Guércio et al., 2006) and the same protein groups found in venom are represented in transcriptome.

The results of such study indicate a large concentration of PIII type which was identified as groups A, C, L, M and P corresponding to jararhagin, bothropasin, berynthractivase, a factor X activator protein from *M. lebetina*, and BOJUMET II homologs in juvenile and sub-adult venom which changed to a large PI-type content in adult venom. Another metalloproteinase groups identified as D and O corresponding to bothrostatin, PII class metalloproteinase from *B. insularis* and Agkistin (Wang et al., 2003). Transcripts assigned as jararhagina, BOJUMET II and bothrostatin, and PII class metalloproteinase from *B. insularis* homologs by Blast searches were observed in our study. On the other hand, we identified some transcripts that encode for metalloproteinases not identified at work as VAPs, halysase, HR1 metalloproteinases.

The level of transcripts encoding PLA_2 enzymes observed in *B. atrox* was higher than in *B. insularis* and *B. jararaca* but lower than in *B. jararacussu*. PLA_2 toxins are commonly present in many isoforms and a single cluster encoded for a non-myotoxic toxin (Asp49 PLA_2) was identified in our dataset. Alape-Giron et al. (2008) observed in *B. asper* venom a shift between Asp49 PLA_2 in juveniles and Lys 49 PLA_2 in adults. Since *B. asper* is phylogenetically close related to *B. atrox* (Castoe and Parkinson, 2006), our results indicate that the same pattern of amino acid replacement may be matched by both snake species. The proteomic characterization grouped in PLA_2 in five groups of which the groups Y and Δ were detected only in juvenile and sub-adult stages and group Y were composed by Asp49 PLA_2.

Others toxins described in the proteomic study as serine proteinases, C-type lectins, L-amino oxidases (LAOs), Cysteine-Rich Secretory Proteins (CRISPs) and vascular endothelial growth factors also were identified in transcriptome analysis. The only clusters encoding LAO and CRISP were the same described on venom proteome.

Concerning serine proteinases detected on venom there was some differences when comparing it with transcripts. From the four groups E, N, Q and R none of which was identified in clusters. This difference could be explained by the high homology of these proteins and may be is difficult to discriminate with employed technique. C-type lectins transcripts encoding Bothrocetin α chain was different from the protein detected on the venom.

For the first time, we describe the detection of an EST in *B. atrox* encoding a phospholipase B-like protein in the venom gland. In addition, an EST encoding a cytosolic phospholipase A2 was also described.

Interestingly, the clustering of C-type lectin ESTs suggests that *B. atrox* venom may exert an inhibitory effect on the coagulation cascade, but this hypothesis needs further clinical support.

Noteworthy, ESTs related to several biochemical pathways related to essential cellular functions were found in *B. atrox* and in other *Bothrops* transcriptomes, what offers worthy information about molecules that might play fundamental role in venom gland function, like toxin processing and fold. Finally, the presence of reverse transcriptase and transposase transcripts suggests the presence of retro-elements in the cells of *B. atrox* venom glands. These elements were identified in others transcriptomes like *Bitis gabonica* (Franscischetti et al., 2004), *Lachesis muta* (Junqueira-de-Azevedo et al., 2006), *D. acutus* (Zhang et al., 2006), *Sistrurus catenatus edwardsii* (Pahari et al., 2007) and are related with the generation of toxin gene diversity. Exon shuffling with these elements has been established as a genetic mechanism for the origin of new genes in invertebrates, vertebrates and plants (Long, 2000; Long et al., 2003). This gene multiplication followed by accelerated Darwinian evolution may represent a mechanism which generates the variability of toxin families.

5. Conclusions

We have provided a catalogue of transcripts commonly expressed in Amazonian *B. atrox* venom gland. Our database constitutes the first collection of ESTs from a pit viper that inhabits the South American rain forest. In this sense, this dataset contributes to support and corroborate proteomic and clinical studies, as well as to comprehend the gene diversity present in the venom gland of snakes of the same species inhabiting distinct geographical regions.

Last but not least, our report will serve as an additional source of information about snake toxin cDNA sequences, which can be used as a comparative tool for other projects related to the molecular phylogenetic and gene expression profile of pit vipers.

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Conflict of interest

The authors declare that there are no conflicts of interests.

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