PROTOCOL DEVELOPING FOR IDENTIFICATION OF VEGETAL MATRICES USED IN AMMODYTES AMMODYTES FREEZE-DRIED VENOM ADULTERATION

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Abstract
Presence of corn flour adulteration was detected by extracting the DNA from 25mg of freeze dried venom and using it as template in PCR amplification with zein specific primers known to be highly specific for corn species. The obtained amplicon was purified from agarose gel and sequenced in order to further confirm the presence of corn specific DNA sequences. The sequence thus obtained was uploaded in a DNA Data Base, and aligned with the reference zein sequence. The 99% of similarity between the two sequences enables us to confirm the corn flour adulteration in the analyzed venom sample.

The decrease of Ammodytes ammodytes venom quality on the Romanian market is strongly felt by producers through increasingly difficult access on the international market.

A quality screening evaluation of this valuable material will lead to a decline of incidence of adulterated sample offered for sale.

Even though in scientific literature, snake venom adulteration is poorly presented (*Calvete et al.. 2015, Inacio et.al. 2016)*, this study was performed as a result of identifying existing needs in the current practice of specialized laboratory in venom analysis from Research Laboratories Horia Cernescu- Banat`s University of Agricultural Sciences and Veterinary Medicine King Michael I of Romania from Timișoara.

Material and methods

Biological material consisted of three freeze dried venom samples noted with A, B and C.

Controls

A sample of pure freeze dried venom was used as negative template control (NTC). Certified reference materials (CRM) - Mon 810 maize produced by the Institute for Reference Materials and Measurements (IRMM) were used as positive template controls (PTC) for vegetal DNA (Hompson et al., 2002; Guidelines on,2010).

DNA extraction

Genomic DNA was extracted from each sample using the CTAB method (ISO 21571, 2005) 25 mg of freeze dried venom was mixed with 300 µl sterile distillated water. 700 µl CTAB buffer (CTAB -20g/l; NaCl- 1,4 M; Tris-HCl - 0,1 M; Na_2EDTA- 20 mM, pH 8) was added together with 20 µl RNase solution (10 mg/ml) and the mixture was incubated at 65 °C for 30 min. The samples were centrifuged at 12,000×g for 10 min and the supernatant was transferred to a tube with 500 µl chloroform, vortexed and centrifuged at 12,000 ×g for 15 min. The upper layer was transferred to a new tube and 2 volumes of CTAB precipitation solution (CTAB – 5g/l; NaCl – 0,04M) were added. The samples were incubated at room temperature for 60 min and centrifuged at 12,000×g for 5 min. The pellet was dissolved in 350 µl NaCl 1.2M and 350 µl cloroform was added. The samples were mixed by vortex and centrifuged at 12,000×g for 10 min. The upper layer was precipitated with 0.6 volumes of isopropanol, incubated at room temperature for 20 min and centrifuged at 12,000 ×g for 10 min. The pellet was washed in 70% ethanol, vacuum dried and re-suspended in 25 µl sterile ultrapure water.

The quality and quantity of DNA was assessed by spectrophotometric method using a

NanoDrop 8000, Thermo Scientific (Glasel, 1995).

Primers used in this study

The presence of vegetal DNA in the samples was assessed by PCR, targeting the chloroplast gene RuBiSco, specific to vegetal genome, with the primers proposed by Rudi et al: CW:5CGTAGCTTCCGGTGGTATCCACGT3', and CX: 5'GGGGCAGGTAAGAAAGGGTTTCGTA3' expected to generate an amplicon of 150 bp.

For detection of maize, the zein gene specific primers were used, with the following sequences 5'ZEIN3: AGTGCGACCCATATTCCAG3' and ZEIN4: 5'GACATTGTGGCATCATCATT3'. The expected fragment length is about 277 bp (*ISO 21569:2005, Hardegger et al., 1999*)

DNA amplification

The final volume for the PCR reactions was 25 µl using Go Taq Green Master Mix PCR kit from Promega according to producer instructions, 20 pmol of primers and 50 ng of DNA template and were performed on a Mastercycler ProS (Eppendorf U.S.) thermocycler.

The amplification program for RuBiSco primers consisted of an initial denaturing step for 3 min at 95°C, followed by 30 cycles of denaturation at 95°C for 20 sec, annealing at 63°C for 45 sec and extension at 72°C for 1 min, with a final step at 72°C for 3 min. The PCR program for lectin primers was: denaturation 95°C - 3 min; 40 cycles: denaturation 95°C -25 sec; primer annealing 62°C - 30 sec, DNA synthesis 72°C - 45 sec; final extension 72°C - 7 min and for the zein primers: denaturation 95°C - 3 min; 40 cycles: denaturation 95°C -30 sec; primer annealing 60°C - 30 sec, DNA synthesis 72°C - 30 sec; final extension 72°C - 3 min.

The resulting PCR products were separated on 2 % agarose gels in TAE buffer at room temperature at a constant voltage of 100 V for 40 minutes. The PCR products were visualized and photographed under UV light (PhotoDocumentation System, UVP, England) *ISO 21569:2005; Mihacea et al.,2009*).

Sequencing reactions

Amplicons were purified from agarose gels using PureLink Quick Gel Extraction & PCR Purification Combo Kit (Invitrogen, Germany) and were send for sequencing to Macrogen Laboratory, Amsterdam ,Holland. The obtained DNA sequences were uploaded in the NCBI Database for the confirmation of identity.

Results and discussions

Total genomic DNA was extracted and purified from the venom samples that were identified as possibly adulterated with corn flour, along with NTC and PTC samples. Even if the available starting material was in low quantity, DNA of amplifiable quality was obtained for all examined samples as revealed by spectrophotometry results (Table 1). At the same time the absorbance ratio A 260/280 and 260/230 was determined.

Table 1

Concentration and quality of DNA isolated from biological material

Sample	DNA quantity in ng/µl	260/280 ratio	260/230 ratio
Freeze dried venom (A)	18	1.57	1.63
Freeze dried venom (B)	16.7	1.64	1.58
Freeze dried venom (C)	19.4	1.48	1.56
PTC (corn flour)	137.3	1.76	2.14
NTC (freeze dried pure venom)	11.3	1.31	1.34

It can be noticed that overall the quality of the samples was appropriate for further analysis, OD 260/280 ratio hovering around 1.31 – 1.76 and OD 260/230 ratio around 1.34 – 2.14.

In order for the template DNA to be at the same concentration for all samples, serial dilutions were carried out so that each sample was 10 ng / µl. Furthermore, all DNA samples were amplified with specific primers for ribulose-bisphosphate carboxylase-1.5 (RuBisCo) gene, this being a reference gene, that is present in all plant cells. Highlighting this gene in a DNA sample extracted from the venom samples is proof of vegetal DNA presence and also of the amplifying quality of this DNA template.

The amplification products corresponding to each sample were analyzed by agarose gel electrophoresis (Figure 1).

Bands of expected size (185 bp) are observable for all analyzed venom samples and also for the positive control. As expected the amplicons are absent in the case of used negative controls: DNA extracted from pure venom sample and the reagents control where no DNA template is inserted. This result emphasizes the fact that the venom samples are adulterated with vegetal material.

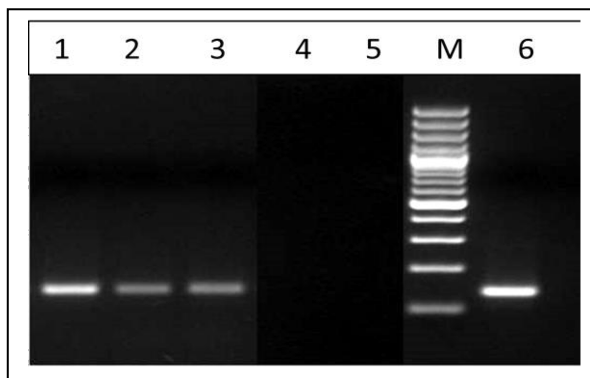

Fig. 1. : Agarose gel electrophoresis of amplification reaction products, specific for ribulose-1,5-bisphosphat carboxylase (RuBisCo) gene : 1. Venom sample A; 2.Venom sample B; 3. Venom sample C; 4. NTC – pure venom sample; 5. Reagents control; M – DNA ladder, Express DNA Ladder (*Fermentas*); 6. PTC – corn flour.

The next step of the analysis was to confirm the venom samples adulteration by detecting the vegetal species that was added. DNA samples were amplified with specific primers for *zein gene* namely oligonucleotides sequences complementary to the DNA sequence specific to corn (Zea mays L.) (Figure 2).

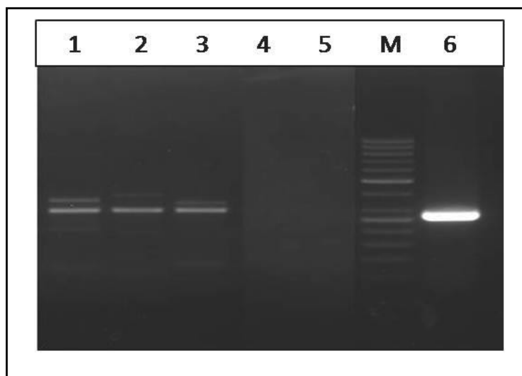

Fig. 2. : Agarose gel elecrophoresis of amplification reaction products, specific for zein gene : 1. Venom sample A; 2.Venom sample B; 3. Venom sample C; 4. NTC – pure venom sample; 5. Reagents control; M – DNA ladder, Express DNA Ladder (*Fermentas*); 6. PTC – corn flour.

The presence of an amplification product with a molecular length of about 277 bp, confirms the presence of corn in all analyzed samples (Moore et al., 2012). Differences between venom samples and positive control amplicons intensity are the proof of adulteration. As expected the venom samples comprise of lower amounts of corn DNA when compared to genuine corn flour. Along with corn specific amplicon, the presence of unspecific amplification products could be detected. Although the used primers are designed to be highly specific for Zea mays L. specie, those unspecific amplification may occur especially when the analyzed samples are of mainly composed of animal material. However, the method only validates as positive result the amplicon that corresponds to expected molecular weight.

For the accuracy of results the obtained amplicons were sequenced. The specific length amplicon was excised from the agarosis gel after the confirmation, purified and subjected to sequencing reaction. Thus obtained sequences were uploaded in NCBI data base and aligned with similar sequences using *blastn* function (Figure 3, 4, 5).

```
>gb|JQ083080.1| Zea mays cultivar Giza 2 10 kD zein protein gene, complete cds
Length=453

 Score =  420 bits (227),  Expect = 2e-113
 Identities = 238/243 (98%), Gaps = 2/243 (1%)
 Strand=Plus/Plus

Query  14   ATGCCCTATAGGTACCATGAACCCATGCATGCAGTACTGCATGATGCAACAGGGGCTTGC  73
            ||||| | | ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94   ATGCCAT-TGGGTACCATGAACCCATGCATGCAGTACTGCATGATGCAACAGGGGCTTGC  152

Query  74   CAGCTTGATGGCGTGTCCGTCCCTGATGCTGCAGCAACTGTTGGCCTTACCGCTTCAGAC  133
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  153  CAGCTTGATGGCGTGTCCGTCCCTGATGCTGCAGCAACTGTTGGCCTTACCGCTTCAGAC  212

Query  134  GATGCCAGTGATGATGCCACAGATGATGACGCCTAACATGATGTCACCATTGATGATGCC  193
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  213  GATGCCAGTGATGATGCCACAGATGATGACGCCTAACATGATGTCACCATTGATGATGCC  272

Query  194  GAGCATGATC-CACCAATGGTCTTGCCGAGCATGATGTCGCAAATGATGATGCCACAATG  252
            |||||||||  |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  273  GAGCATGATGTCACCAATGGTCTTGCCGAGCATGATGTCGCAAATGATGATGCCACAATG  332

Query  253  TCA  255
            |||
Sbjct  333  TCA  335
```

Figure 3 : Alignment of the venom sample A DNA sequence with zein gene sequence from NCBI Data Base

In the case of venom sample A, reliable DNA sequence could be considered only for 255 base pairs, the rest of the sequence being undefined. This 255 base pair sequence was found to have 98% similarity with the specific zein gene sequence from the NCBI Data Base.

```
>gb|JQ083080.1| Zea mays cultivar Giza 2 10 kD zein protein gene, complete cds
Length=453

 Score =  425 bits (230),  Expect = 3e-115
 Identities = 232/233 (99%), Gaps = 0/233 (0%)
 Strand=Plus/Plus

Query  24   GTACCATGAACCCATGCATGCAGTACTGCATGATGCAACAGGGGCTTGCCAGCTTGATGG  83
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  104  GTACCATGAACCCATGCATGCAGTACTGCATGATGCAACAGGGGCTTGCCAGCTTGATGG  163

Query  84   CGTGTCCGTCCCTGATGCTGCAGCAACTGTTGGCCTTACCGCTTCAGACGATGCCAGTGA  143
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  164  CGTGTCCGTCCCTGATGCTGCAGCAACTGTTGGCCTTACCGCTTCAGACGATGCCAGTGA  223

Query  144  TGATGCCACAGATGATGACGCCTAACATGATGTCACCATTGATGATGCCGAGCATGATGT  203
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224  TGATGCCACAGATGATGACGCCTAACATGATGTCACCATTGATGATGCCGAGCATGATGT  283

Query  204  CACCAATGGTCTTGCTGAGCATGATGTCGCAAATGATGATGCCACAATGTCAC  256
            ||||||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct  284  CACCAATGGTCTTGCCGAGCATGATGTCGCAAATGATGATGCCACAATGTCAC  336
```

Figure 4 : Alignment of the venom sample B DNA sequence with zein gene sequence from NCBI Data Base

For the venom sample B, only for 233 of base pairs could be properly sequenced, the rest of the sequence being undefined. The 233 base pair sequence proved to be similar in 99 % with the specific zein gene sequence from the NCBI Data Base.

```
>gb|JQ083080.1| Zea mays cultivar Giza 2 10 kD zein protein gene, complete cds
Length=453

 Score =  420 bits (227),  Expect = 2e-113
 Identities = 238/243 (98%), Gaps = 2/243 (1%)
 Strand=Plus/Plus

Query  14   ATGCCCTATAGGTACCATGAACCCATGCATGCAGTACTGCATGATGCAACAGGGGCTTGC  73
            ||||| | | ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94   ATGCCAT-TGGGTACCATGAACCCATGCATGCAGTACTGCATGATGCAACAGGGGCTTGC  152

Query  74   CAGCTTGATGGCGTGTCCGTCCCTGATGCTGCAGCAACTGTTGGCCTTACCGCTTCAGAC  133
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  153  CAGCTTGATGGCGTGTCCGTCCCTGATGCTGCAGCAACTGTTGGCCTTACCGCTTCAGAC  212

Query  134  GATGCCAGTGATGATGCCACAGATGATGACGCCTAACATGATGTCACCATTGATGATGCC  193
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  213  GATGCCAGTGATGATGCCACAGATGATGACGCCTAACATGATGTCACCATTGATGATGCC  272

Query  194  GAGCATGATC-CACCAATGGTCTTGCCGAGCATGATGTCGCAAATGATGATGCCACAATG  252
            |||||||||  |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  273  GAGCATGATGTCACCAATGGTCTTGCCGAGCATGATGTCGCAAATGATGATGCCACAATG  332

Query  253  TCA  255
            |||
Sbjct  333  TCA  335
```

Figure 5 : Alignment of the venom sample C DNA sequence with zein gene sequence from NCBI Data Base

Similar to venom sample A, in the case of venom sample C, 233 of base pairs could be properly sequenced, the rest of the sequence being difficult to define. This 255 base pair sequence was found to have also 98% similarity with the specific zein gene sequence from the NCBI Data Base.

Conclusions

Developing a protocol for Ammodytes ammodytes freeze dried venom adulteration with vegetal matrices using genomic techniques could represent an important step in screening evaluation of venom quality and will be a helpful tool in increasing the quality of raw material from Romanian venom market.

Aknowlegdements

The present research was carried in the Antioxidant Systems Research Laboratory (A1C) and Molecular Biology Research Laboratory (A2) from the Horia Cernescu Research Unit established through ***POSCCE SMIS 2669 project,*** *in the frame of the research project no. 181/14.01.2016 -* ***Protocol development for evaluating the quality of snake venom in different conditioning form.***

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