Can crickets replace mice in the venom toxicity test?

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INTRODUCTION

Venomous snakes use their venoms (complex mixtures of enymes and non-enzymatic proteins) to restrain and immobilize the prey before ingestion. It is known that determination of 50% lethal doses (LD_{50}) in mice appears to be an important step to assess (and compare) venom's toxic activity. An overwhelming majority of snakes are predators, which feed on different taxa of animals, ranging from arthropods to mammals.

There are three species of venomous snakes in Croatia: long nosed viper (*Vipera ammodytes ammodytes – Vaa*), karst (meadow) viper (*Vipera ursinii* ssp. – *VuCro*) and adder with two of subspecies - European adder (*Vipera berus berus – Vbb*) and Bosnian adder (*Vipera berus bosniensis – Vbbos).* Their eating habits are different. *Va* and *Vb* primarily eat small mammals. The karst viper in Croatia (*VuCro*) favours high-mountain dry grasslands so its choice of food is limited on insects.

MATERIALS AND METHODS

SNAKE VENOMS AND ANIMALS *VuCRO* venom was a pooled sample obtained by milking of >10 adult snakes caught at the southern Velebit isolated locality and released afterwards. It was air dried and stored in the dark at 4 °C until use. Air-dried crude *Vaa* venom was from Institute of Immunology Inc., Croatia. Commercial freeze-dried *Vipera berus berus* (*Vbb)* venom (Serpentarium of the Central Trade Base „Zoo-obyedinenie" Khimky, Moscow District, Russia) was the generous gift of Dr. Juri Siigur from NICPB, Tallinn, Estonia.
Mice and rats used for *in vivo* assays were bred at the Institute of Immunology. Procedures, handling and animal work were in accordance to the Croatian Law on Animal Welfare (2013) which complies with EC Directive 2010/63/EU. Crickets are purchased from a local dealer.

MEASUREMENT OF LETHAL TOXICITY IN MICE
The lethal toxicity expressed as the median lethal dose (LD_{50}) was determined according to the European Pharmacopeia (Ph.Eur. 01/2008:0145). The minimum haemorrhagic dose (MHD) of venom is defined as the least amount of venom (in mg of dry mass) which, when injected intradermally into rats, results in haemorrhagic lesion of 10 mm diameter 24 h later and was determined as described in Lang Balija *et al.* (2005). All results are given as mean from at least three determinations ± standard error (SE).
MEASUREMENT OF LETHAL TOXICITY IN CRICKETS
To demonstrate the potential entomotoxic effect of *VuCro* in comparison to *Vaa* venom, we performed a lethal toxicity test on cricket as described in Starkov *et al.* (2007). The toxicity determination was performed using cricket *Gryllus assimilis*. Groups of 4 crickets weighing 0,50 – 0,95 g were injected with 5 µL of respective venom solutions serially diluted with DF = 2 in water for injection (WFI). Doses ranged from 1,56 to 100 µg/per cricket. An equal volume of WFI (5 µL) was injected to control group of insects. Percent mortality was recorded 2, 24 and 48 h after injection. The median lethal dose was calculated by Probit method. The results are given as the mean ± standard error (MEAN± SE).
ELECTROPHORESIS, PROTEIN DETECTION AND IDENTIFICATION
SDS-PAGE analysis was performed using Bis-Tris precast gels (4-12%) according to manufacturer's instructions (Invitrogen, ThermoFisher Scientific). Detection of proteins was performed using Coomassie Brilliant Blue R250.

conclusion

Venom lethal toxicity assay in mice causes suffering, pain and death of the experimental animals and also requires a large number of animals. This test has been identified by ECVAM (European Centre for Validation of Alternative Methods) as assay that should be necessarily replaced with alternative methods.

In our study we successfully established this test in crickets and compared it with the assay in mice. All venoms were lethally toxic in both experimental animals. However, the higher toxicity of the venom to the preferred prey of the snake species was demonstrated, since small rodents (mostly mice) are preferential diet for other Croatian vipers, while VuCro includes dominantly insects in its food.

The lethal venom toxicity test in crickets is accordant with 3R principles, but can not simply replace the test in mice. However, assay in cricket might be extremely suitable for studies on entomophageous venoms composition and function.

RESULTS AND DISCUSSION

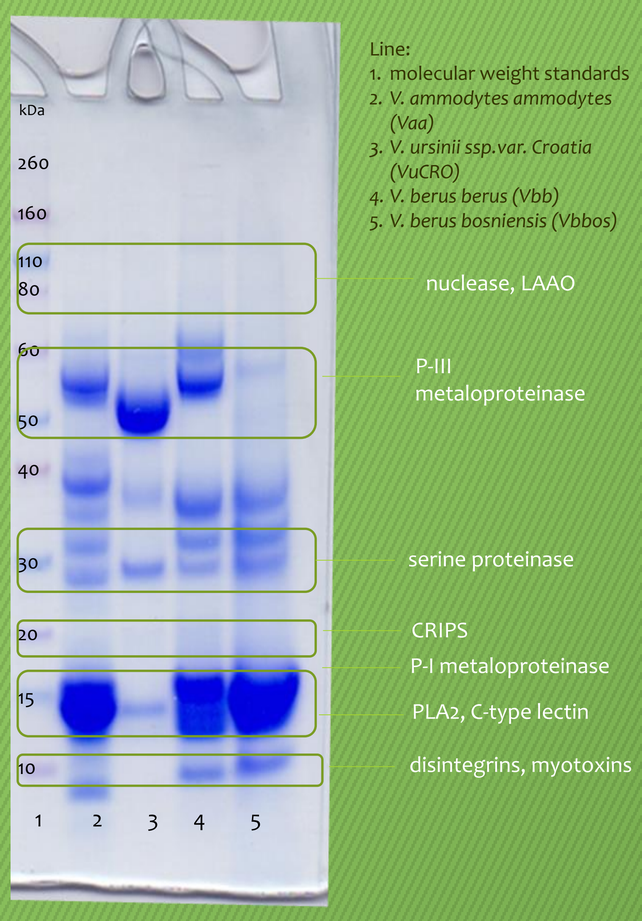

Figure 1. SDS-PAGE analysis of different snake venoms from Croatia under reducing conditions. Each lane contains 40 µg of venom. Basic protein families are marked according to Maskessy S.P. (2010).

Table 1. The difference in toxicity depending on the test animal – lethal toxicity (LD50 in µg) measured in mice or cricket, hemorrhagic activity (MHD in µg) measured in rats and average amount of venom yield of venomous snakes in Croatia; 1 - depending on the geografical location (as determined by Halassy *et al*, 2011.); 2 - measured only for the collection sample from the Poštak; 3 - measured for in house standard of the Institute of Immunology Inc., Zagreb; 4 - measured only for pooled venom samples of venom from location Šumeće, Slavonski Brod

	V. ammodytes ammodytes	*V. ursinii ssp. (Cro)*	*V. berus berus*	*V. berus bosniensis*
distribution in Croatia	the whole Mediterranean part, Gorski Kotar, Lika, Kordun, SW Croatia - south slopes of Žumberak and Samobor hills, Medvednica, Strahinjščica, Ivanščica and Kalnik, Croatian Zagorje and Istria; Islands - only confirmed on Krk, Pag, Vir, Brač, Hvar, Korčula and Mljet	strictly limited locations: South Velebit, Poštak, Lisac, Dinara, Troglav and Kamešnica	the mountainous regions of Gorski Kotar and probably on the Velika and Mala Kapela	the lowlands of Croatia, in the lowlands of the large rivers(Sava, Drava, Mura and the Danube)
LD_{50} (µg) Mouse	4,4-13,7[1]	37,01 ± 0,05 (n=3) [2]	11,1-12,9[3]	9,15-11,1[4]
LD_{50} (µg) Cricket	38,7 ± 3,3 (n=2)	7,1 ± 0,4 (n=2)	75,1	94,3
MHD (µg)	21,6-42,8	34,12 ± 4,75 (n=4)	> 12	> 50
venom yield (mg)	10-45	0,5-4	4-10	4-10

Vum venom is less lethally toxic in mice than *Vaa* venom (Table 1). *VuCro* venom toxicity in crickets was more than 5 times higher than toxicity of *Vaa* venom, 10 times higher than *Vbb* and 13,5 times higher than *Vbbos.* In contrast, *VuCro* venom in mice was more than 4 times less toxic than other venoms. However, the pattern of mice dying indicated the presence of a strong neurotoxic component in *VuCro* venom. Interestingly, SDS-PAGE (Figure 1) revealed a lack of neurotoxic PLA2 proteins, like in other venoms (band at 15 kDa). Metalloproteinases are the most abundant components of *VuCro* venom. Accordingly, *VuCro* venom exhibited strong haemorrhagic activity, comparable to that of the other Croatian viper venoms (Table 1). Taken all together, *VuCro* venom might be a good starting material for the discovery of a novel neurotoxic component in *Vipera* venoms with potentially insecticidal activity. So test on cricket will help us to discover a component which makes those venoms different.

Literature:
Halassy *et al.* Comp. Biochem. Physiol. C 153 (2011) 223–230
Lang Balija *et al.* Comp. Biochem. Physiol. C 140 (2005) 257–263
Maskessy S.P. (2010) Handbook of venoms and Toxins of Reptiles, Taylor&Francis, Boca Raton, London, New York
Starkov *et al.* Toxicon 49 (2007) 995-1001

This work was fully financially supported by Croatian Science Foundation (IP-2014-09-4915). Any opinions, findings or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of Croatian Science Foundation.