ABC transporters are not involved in the detoxification of *Azadirachta indica* extracts in *Anopheles stephensi* larvae.

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Abstract

Objective: detoxifying pathways of mosquitoes against neem extracts are still unclear. The aim of the present study is to investigate the role of ABC-transporters in this process in *Anopheles stephensi,* one of the main malaria vectors in southern Asia.

Methods: third instar larvae of *An. stephens*i were fed with fish food alone or in combination with neem extract at 0.5, 1, 5 and 10%. Six ABC-transporter genes from three different subfamilies (B, C and G) have been analysed to assess relative expression compared to the control. A bioassay was also performed to assess larval mortality rate at the different concentrations in combination with verapamil, an ABC-transporter inhibitor.

Results: The use of verapamil, an ABC transporter inhibitor did not induce an increase of mortality at any of the tested neem extract doses. Furthermore, no significant variation in the expression levels of any transporter belonging to the B, C and G subfamilies was detected.

Conclusion: ABC transporters are not involved in response/defence to Neem extract, differently by the treatments with permethrin, as seen in other works.

Keywords

mosquito defences, natural insecticides, neem tree, vector control, detoxification

Introduction

Malaria is one of the main problems in developing countries. About 212 million new cases occurred in 2015, with 429000 deaths [1] and millions of people which do not receive the services they need.

The use of long lasting insecticidal nets (LLINs), artemisinin-based therapies and indoor residual spraying (IRS) are the main interventions aimed to prevent malaria infections and spread. Vector control through insecticides is a core component of malaria control programmes, but the continuous use of chemical compounds led to resistance insurgence in different vector populations that threaten the global malaria control efforts [2, 3, 4]. Of the 73 malaria endemic countries providing data to the WHO, 60 reported resistance to at least one insecticide class, while 50 reported resistance to two or three classes (WHO, 2016). For this reason, research in alternative insecticides of botanical origin has grown in last decades. In particular *Azadirachta indica*, also known as neem tree, has been used for centuries in traditional medicine [5, 6]. This was probably due to the wide effects that this plant has on parasites and other agents of infection [6]. *A. Indica* and other Meliaceae species have shown a strong larvicidal, anti-emergence, repellency, anti-oviposition effect in different mosquito species [7, 8, 9, 10, 11, 12, 13, 14]. In addition, products based on Neem rarely induce resistance thanks to their wide mode of action [15]. It is important to investigate the detoxifying mechanisms against Neem to understand whether there is a risk of resistance insurgence, noticed also for other insecticides. It is now known that ATP-Binding Cassette (ABC) transporters are involved in the detoxification process of several compounds in different mosquito species such as *An. stephensi* [16-20], *An. gambiae* [21], *Aedes aegypti* [22], *Ae. Albopictus* [23]. In particular on *An. stephensi* it has been demonstrated that, among the eight sub-families of ABC transporters existing in insects, the B and G sub-families play a major role in the detoxification against permethrin, showing a pattern of response that varies with time [16, 17, 19, 20]. Despite their importance against pyrethroids, these genes are not differentially expressed in response to larval exposure to temephos, a widely used larvicide, highlighting an insecticide-specific involvement of the transporters in this mosquito species [18]. For these reasons, the goal of our study is to thoroughly investigate the implication of ABC-transporters in *An. stephensi* defence against neem.

Material and methods.

Bioassay

All mosquitoes used in this study derived from a susceptible *Anopheles stephensi* Liston colony of the insectary of the University of Camerino, Italy, maintained at standard conditions (28±1°C, 85% humidity, 12:12 L-D) and fed with fish food (Tetra, Melle, Germany). Third instar larvae were used for bioassays and molecular analysis, according to the protocols described in Epis et al. [16,17]. Experimental groups were fed with fish food (FF) containing neem seed extract (FF + neem) at different concentrations: 0.5, 1, 5 and 10%. To obtain these concentrations, 1g FF was homogenized to neem extract in 50ml chloroform (Sigma-Aldrich), mixed for 10 minutes and then evaporated at

reduced pressure (37°C, 3 mmHg) with a Büchi R 200 rotavapor. The powder obtained was left at room temperature for 24h.

For the bioassay, five groups of 25 third instar larvae were put in 100 ml of spring water and fed with FF + neem at different concentrations (0, 0.5, 5 and 10%), alone or in combination with a sub-lethal dose of the inhibitor verapamil, as reported in previous works [16, 17]. The control groups were fed just with FF. Two additional groups, fed with FF and treated with verapamil, were used as control group. Mortality was assessed every 24h for three days.

To investigate the effect of different treatments on the larval mortality, we run a Generalized Linear Mixed Model (GLMM) with Poisson error structure, using the number of dead larvae as dependent variable and considering replicates as a residual-type random component. We explored the effect on the response variable of dose/concentration of insecticide (i.e. 0%, 0.5%, 1%, 5%, 10%), addition of verapamil (i.e. yes or no), time after treatment (i.e. 24, 48 or 72 hours) and dose by verapamil interaction. The initial number of larvae of each replicate was included in the model as a covariate. Interactions were excluded from the final model when not significant. Interpretation of effects with more than two levels was based on pair-wise t-tests of Differences of Least Square Means (DLSM), applying Tukey correction for multiple comparisons. The analysis were carried out through PROCGLIMMIX in SAS/STAT9.4 software (Copyright © 2002--2012, SAS Institute Inc., Cary, NC, USA).

Expression profile after insecticide treatment

The six genes analysed, encoding for ABC transporters (*Anst*ABCB2, *Anst*ABCB3, *Anst*ABCB4, *Anst*ABCBmember6, *Anst*ABCG4, *Anst*ABCC11) in *An. stephensi*, were chosen due to their involvement in the defence against the insecticide permethrin. The expression profile of these genes was evaluated in larvae after 0.5h, 24h, 48h and 72h of treatment at different Neem concentration. RNA extraction, cDNA synthesis, quantitative RT-PCRs were performed following the protocol described in detail in [16, 17]. Two different genes, RPS7 and GAPDH, have been used as reference genes to normalize relative expression. The primers used in this work are reported in table 1. To detect any significant effect of neem treatment on the expression of ABC genes, RT-PCR data were analysed trough non-parametric Wilcoxon two-sample tests, due to the non-normal distribution of some samples [24]. For each of the six genes and each of the dose-time combinations, we compared differences in ΔCT (CT_{target} - $CT_{housekeeping}$) between treated and control (i.e. dose 0) samples. Estimates of $\Delta\Delta CT$ values and their 95% confidence limits were obtained through the Hodges-Lehman method. All the analysis were carried out using PROC NPAR1WAY in SAS® 9.4 Software (Copyright © 2012 SAS Institute Inc., Cary, NC, USA).

Results and discussion

Bioassay

Verapamil concentration was established according to a previous work on ABC-transporter in *An. stephensi* [16]. The bioassay confirms, in this species, that neem extracts have larvicidal effects, as seen by other authors [7, 8, 11, 24]. At the same time, the results of insecticide exposure in combination with verapamil demonstrate that ABC transporters are not involved in the cellular response of *An. stephensi* against this toxicant (fig.1), in contrast with the effect of other insecticide tests [16] in which verapamil, added to permethrin treatment, induced an increased mortality compared to the insecticide alone.

Mortality of larvae increased significantly with time ($F_{2, 18}=41.4$; $p<0.0001$) and at higher doses/concentrations of insecticide ($F_{4, 36}=16.8$; $p<0.0001$). Addition of verapamil had no effect on larvae mortality, either as a single factor or in interaction with insecticide (both $p>0.13$).

Expression profile after insecticide treatment

Statistical analysis of RT-PCR data (tab.2) did not reveal any effect of neem treatment on ABC genes expression: ΔCT values of treated samples were not significantly different from controls, for any of the 6 target genes and any of the dose-time combinations (all $p>0.05$). This outcome is in line with data reported in Porretta et al. [18] that tested the insecticide temephos against *An. stephensi*, obtaining similar results and indicating that different compounds can induce different responses of the mosquito ABC transporters. This work cannot exclude the implication of other detoxification mechanisms and, for this reason, further investigations are needed to clarify and amplify the set of transporters analysed, taking into account also different metabolic pathways that could be involved. These results are important in an attempt of widen the global knowledge on the detoxification from xenobiotics in mosquito *An. stephensi*.

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204 **Table 1** Primer sequences of ABC transporters and housekeeping genes of *Anopheles stephensi*.

Gene	Forward primer	Reverse primer	bp	Source
*Anst*ABCB2	TATCAAGTTCACGGATGTAGAGT	TATCCACCTTGCCACTGTC	185	[16]
*Anst*ABCB3	CAACCGTTCCGTAATACTACC	ACTGGTAGCCCAATGTGAAG	133	[16]
*Anst*ABCB4	GGACAAAACATTCGGGAGG	CGTAGTGAATGTTGTGGCG	109	[16]
*Anst*ABCBmember6	CTGGAGACGCTGAGAGATA	TACTCCTCGGTGAACTGG	125	[16]
*Anst*ABCC11	GGTTGGATTGGCTTTCGTG	ATAACCGACTCCCGTTTCG	156	[17]
*Anst*ABCG4	ATGAGCCCATTCGTCCTG	AGCGTGGAGAAGAAGCAG	158	[16]
RPS7	AGCAGCAGCAGCACTTGATTTG	TAAACGGCTTTCTGCGTCACCC	90	[26]
GAPDH	GCCGTCGGCAAGGTCATCCC	TTCATCGGTCCGTTGGCGGC	166	[27]

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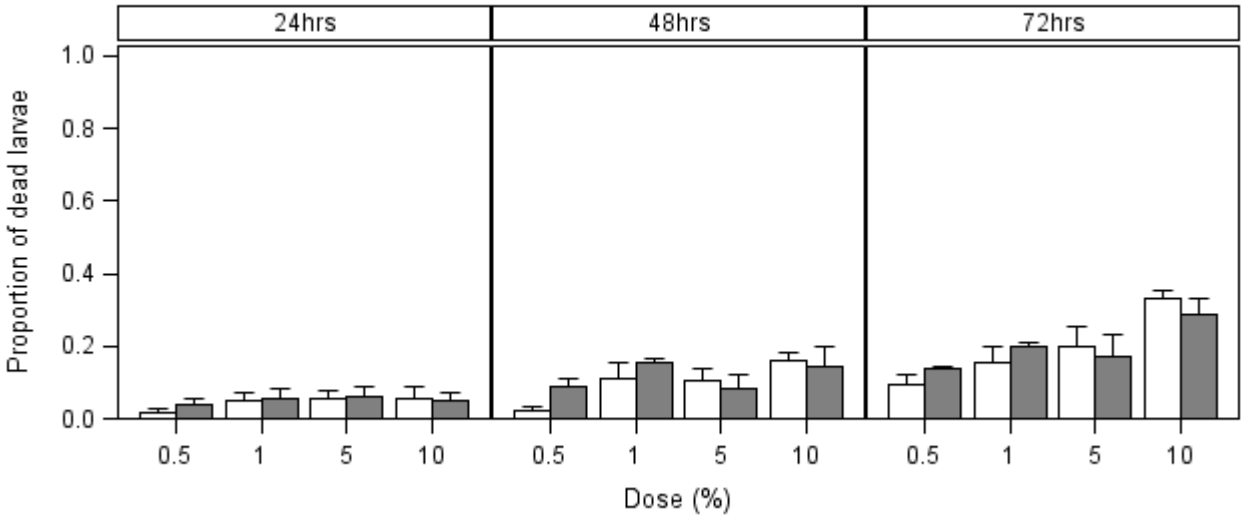
Table 2 Relative expression of *Anopheles stephensi* ABC genes obtained with quantitative RT-PCR after treatment with neem extract at different times. Expression level of the control, non-treated larvae, was considered to be the basal level (equal 1). RPS7 and GAPDH were used as reference genes to normalize expression levels. The values are expressed as mean ± standard error.

Exposure time	Insecticide concentration	*Anst*ABCB2	*Anst*ABCB3	*Anst*ABCB4	*Anst*ABCB member6	*Anst*ABCC11	*Anst*ABCG4
0.5h	0.5%	1,43 ± 0,11	1,37 ± 0,19	1,39 ± 0,35	1,39 ± 0,16	1,16 ± 0,09	1,28 ± 0,70
	1%	0,98± 0,29	0,78± 0,27	1,13 ± 0.39	0,78 ± 0,28	0,93 ± 0,21	1,46 ± 0,92
	5%	1,95± 0,70	1,29 ± 0,51	1,11 ± 0,45	1,32 ± 0,45	0,77 ± 0,18	1,02 ± 0,79
	10%	2,74± 0.57	1,66 ± 0,36	1,21 ± 0,22	2,08 ± 0,44	0,96 ± 0,13	1,98 ± 1,42
24 h	0.5%	1,25 ± 0,08	1,29 ± 0,14	1,31 ± 0,18	1,32 ± 0,20	0,95 ± 0,04	1,06 ± 0,31
	1%	1,28 ± 0,09	1,47 ± 0,26	1,39 ± 0,32	1,32 ± 0,28	1,12 ± 0,07	2,26 ± 0,93
	5%	0,81 ± 0,09	0,55 ± 0,08	0,66 ± 0,07	0,98 ± 0,16	0,95 ± 0,13	1,62 ± 0,61
	10%	1,15 ± 0,14	0,87 ± 027	1,11 ± 0,15	1,06 ± 0,24	1,07 ± 0,15	1,55 ± 0,46
48 h	0.5%	0,62 ± 0,26	0,59 ± 0,13	0,47 ± 0,10	0,66 ± 0,19	1,25 ± 0,24	0,80 ± 0,36
	1%	1,05 ± 0,27	0,79 ± 0,12	0,76 ± 0,10	0,85 ± 0,22	1,13 ± 0,06	1,42 ± 0,73
	5%	0,87 ± 0,22	0,92 ± 0,35	0,67 ± 0,19	0,85 ± 0,18	0,88 ± 0,14	2,55 ± 1,06
	10%	1,13 ± 0,60	0,50 ± 0,12	0,46 ± 0,03	0,54 ± 0,16	0,97 ± 0,03	2,24 ± 1,60
72 h	0.5%	0,49 ± 0,06	0,57 ± 0,13	0,88 ± 0,10	0,82 ± 0,09	0,86 ± 0,10	1,35 ± 0,21
	1%	0,50 ± 0,27	0,58 ± 0,20	0,80 ± 0,08	0,88 ± 0,21	0,93 ± 0,04	2,13 ± 0,31
	5%	0,55 ± 0,01	0,59 ± 0,19	1,02 ± 0,03	0,93 ± 0,20	0,59 ± 0,09	2,23 ± 0,33
	10%	0,73 ± 0,10	0,61 ± 0,14	1,13 ± 0,11	1,05 ± 0,23	0,84 ± 0,04	2,55 ± 0,37

213 Figure 1. Proportion of dead larvae at different times and insecticide concentrations, with (white bars) and without (grey
214 bars) verapamil addition. Error bars indicate standard errors.



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