SCIENTIFIC NOTE

ADENOSINE TRIPHOSPHATE–BINDING CASSETTE TRANSPORTERS ARE NOT INVOLVED IN THE DETOXIFICATION OF AZADIRACHTA INDICA EXTRACTS IN ANOPHELES STEPHENSI LARVAE

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ABSTRACT. Detoxifying pathways of mosquitoes against the neem (Azadirachta indica) extracts are still unclear. The aim of the present study was to investigate the role of adenosine triphosphate–binding cassette (ABC) transporters in this process in Anopheles stephensi, one of the main malaria vectors in southern Asia. Third-stage larvae of An. stephensi were fed with fish food alone or in combination with neem extract at 0.5%, 1%, 5%, and 10%. Six ABC-transporter genes from 3 different subfamilies (B, C, and G) were analyzed to assess their relative expression compared with controls. A bioassay was also performed to assess larval mortality rate at different concentrations and in combination with verapamil, an ABC-transporter inhibitor. No significant variation in the expression levels of any transporter belonging to the B, C, and G subfamilies was detected. Furthermore, the use of verapamil did not induce an increase in mortality at any of the tested neem extract concentrations, indicating that ABC transporters are not involved in the detoxification of neem extracts in An. stephensi larvae.

KEY WORDS Detoxification, mosquito defenses, natural insecticides, neem tree, vector control

Malaria is a major health problem in developing countries. According to the World Health Organization, about 216 million cases occurred in 2016, with 445,000 deaths (WHO 2017). Long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), and artemisinin-based therapies are the main interventions aimed at preventing malaria infection and spread. Vector control through insecticides is a core component of malaria control programs, but the continuous use of chemical compounds has led to the emergence of resistance in different vector populations, thus threatening the global malaria control efforts (Alonso and Tanner 2013). Out of the 73 malaria-endemic countries providing data to the WHO, 60 reported resistance to at least 1 insecticide class, while 50 reported resistance to 2 or 3 classes (WHO 2017). For these reasons, the objective of this study was to thoroughly investigate the potential role of ABC transporters in An. stephensi defense against the neem extract.

All the mosquitoes used in this study derived from a susceptible An. stephensi colony held at the insectary of the University of Camerino, Italy. The
A. indica seeds were crushed and homogenized to 1 g FF in 50 ml chloroform (Sigma-Aldrich, St. Louis, MO), mixed for 10 min, and then evaporated at a reduced pressure (37°C, 3 mmHg) with a Büchi R 200 rotavapor. The powder obtained was left at room temperature for 24 h.

For the bioassay, 5 groups of on average 25 3rd instars (range: 24–28) 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 sublethal dose of the inhibitor verapamil (100 μM), as reported in previous studies (Epis et al. 2014a, 2014b). Verapamil is a blocker of calcium channels that competes with toxic compounds for the extrusion by transmembrane pumps. Control groups with FF alone or verapamil with FF were included. Mortality was assessed every 24 h for 3 days. To investigate the effect of different treatments on larval mortality, we ran a generalized linear mixed model 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 the concentration of neem extract (i.e., 0%, 0.5%, 1%, 5%, 10%), addition of verapamil (yes/no), time of treatments (24, 48, or 72 h) and their 2nd-order interactions. The initial number of larvae of each replicate was included in the model as a covariate. Interactions were excluded from the final model account were down-regulated or not differentially expressed at the different time points (Epis et al. 2014a) used verapamil in combination with permethrin to demonstrate ABC’s involvement against pyrethroids. On the other side, Epis et al. (2014a) used verapamil in combination with permethrin to demonstrate ABC’s involvement against pyrethroids. The combined treatment could lower the 50% lethal dose from 0.137 mg/liter (permethrin alone) to 0.025 mg/liter (permethrin + verapamil). Their mortality results were supported by RT-PCR data, showing a differential expression of the genes analyzed, in particular ABCG4 and ABCmember6. The overexpression peak of these 2 genes was detected after 6 h exposure, but the up-regulation persisted after 24 h. The other genes taken into account were down-regulated or not differentially expressed at the different time points (Epis et al. 2014a, 2014b; De Marco et al. 2017). In the present study, the analysis of RT-PCR data did not reveal any effect of neem treatment on ABC genes’ expression: treated sample ΔCt values were not significantly different from controls, for any of the 6 target genes and any of the dose–time combinations (all $P > 0.05$). Gene expression analysis confirmed the
bioassay data, demonstrating that ABC transporters were not involved in the cellular response of *An. stephensi* to neem extracts. Also, similar expression results were shown by Porretta et al. (2016), where none of the investigated genes were differentially expressed after temephos exposure. All together, these results indicate that different compounds can induce different responses in the *An. stephensi* ABC transporters.

In conclusion, the present study demonstrates that the analyzed ABC transporters are not involved in response/defense to neem extracts in *An. stephensi* larvae. However, we cannot exclude other mechanisms involved in neem extract’s detoxification, and, for this reason, further investigations are needed to clarify the response of *An. stephensi*. In particular, future studies should focus on phase I and phase II detoxification enzymes, such as cytochrome P450, carboxylesterases, UDP-glucuronosyltransferases, and glutathione S-transferases, known to be differentially expressed in response to various xenobiotics used for vector control.

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