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An insight into the sialome of the adult female mosquito *Aedes albopictus*

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Abstract

To gain insight into the molecular repertoire of the adult female salivary glands of the tiger mosquito *Aedes albopictus*, we performed transcriptome and proteome analysis. cDNA clones were sequenced and assembled in clusters of related sequences and the corresponding genes assigned to one of three categories: housekeeping (H; 31%), secreted (S; 34%), or unknown (U; 35%) function. Among the putative secreted factors are proteins known to be widely distributed in the saliva of blood-sucking Diptera, such as D7 and antigen 5 family members, as well as proteins that are mosquito- or culicine-specific, i.e., the 30-kDa allergen or the 62-kDa and 34-kDa families, respectively. Expression of 15 of these salivary proteins was confirmed by Edman degradation. Tissue and sex specificity of selected transcripts were evaluated by RT-PCR and identified at least 32 genes whose expression is restricted or enriched in the female salivary glands of *Ae. albopictus*, whereas 17 additional genes were expressed in female glands and adult males but not in other tissues of adult females. For approximately one third of the genes analyzed, involvement in blood-feeding, sugar digestion, immune response, or other more generic physiological roles can be postulated; however, no functions can be suggested for the remaining sequences, which therefore likely represent either novel functions or novel molecules recruited during the evolution of hematophagy. Supplemental spreadsheets with hyperlinks to all sequences used in this manuscript are hyperlinked throughout the text and can be found at http://www.ncbi.nlm.nih.gov/projects/omes/#salivarytranscriptomes.
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1. Introduction

The tiger mosquito *Aedes albopictus* is probably the fastest-spreading mosquito in the world. Historically original to tropical forests of Southeast Asia, it was already present in a large number of islands of the western Pacific and Indian Oceans in the middle of 20th century. Amazingly, in the last two decades, *Ae. albopictus* attained an almost global distribution, being presently found in the United States, Central and South America (as far south as Argentina), Africa, Europe, and the Middle East. Several reasons contributed to the rapid spreading of this mosquito species; among the most important, certainly, are the resistance of its desiccated, dormant eggs and their efficient passive transport around the world, often in used tires (Tatem et al., 2006). Moreover, a significant contribution to the current widespread distribution came from the ability of *Ae. albopictus* populations to survive to relatively low temperatures (10 °C or lower) and to exploit for oviposition a large variety of breeding sites originated by human activities.

Ae. albopictus is an efficient laboratory vector for a large number of arboviruses (dengue, yellow fever, West Nile,

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and several others), and its rapid global spreading has thus raised concern among public health officials; however, with the exception of some small outbreaks of dengue fever, its efficiency as a vector for arboviral diseases seems to be important in nature as suggested by laboratory data (Gratz, 2004; Gubler, 2003). After the first documented introduction in Genoa in 1990 (Sabatini et al., 1990), *Ae. albopictus* became well established in Italy. Thanks to some of its ecological characteristics (daylight biting habit, high density, aggressive and anthropophilic behavior), it became a major biting pest (Romi, 2001) and, as recently suggested, a possible natural vector of the filarial nematode *Dirofilaria immitis* (Cancrini et al., 2003).

The salivary glands of blood-feeding arthropods secrete a large number of bioactive molecules essential for efficient blood feeding; among these the most well known are certainly anti-hemostatics, but anti-inflammatory and immunomodulatory agents are also present (Ribeiro and Francischetti, 2003; Ribeiro, 1995). In the last few years, salivary gland transcriptome analyses have highlighted the complexity of mosquito salivary secretions, pointing out how little we know concerning targets and functional roles of salivary components (Arca et al., 2005; Calvo et al., 2004; Ribeiro et al., 2004; Valenzuela et al., 2002b, 2003). Salivary proteins injected into the host skin during feeding may also act as antigens and/or allergens, inducing host immune reactions. In this respect, *Ae. albopictus* may induce intense local cutaneous reactions and has been reported as the most common species associated with severe systemic allergic reaction to mosquito bites (Peng et al., 2004). Moreover, immunoblot analysis of salivary extracts with sera from individuals allergic to mosquitoes indicated that *Ae. albopictus* saliva carries at least 16 different allergens, the highest number among the 10 mosquito species examined (Peng and Simons, 2004). In view of its possible role as a disease vector and because of the unique allergenic potential of its salivary protein repertoire, we have begun transcriptome analysis of the salivary glands of the tiger mosquito *Ae. albopictus*, and we report here 69 novel cDNA's encoding putative salivary-secreted proteins.

2. Methods

2.1. Mosquitoes

The *Ae. albopictus* strain used in this study (kindly provided by R. Romi, Istituto Superiore di Sanità) was caught in Rome in 2000 and reared under standard laboratory conditions ($25 \pm 1\,^{\circ}C$, relative humidity $60 \pm 10\%$, light:dark photoperiod 14:10 h) in the insectary of the Department of Public Health at University La Sapienza, Rome. Adult females (1–5 days old) maintained on a 10% sucrose diet were used in this study. Salivary glands were dissected, collected in groups of 20 in 20 µl of PBS buffer (150 mM NaCl, 5 mM Na-phosphate, pH 7.4), frozen in liquid nitrogen, and stored at $-80\,^{\circ}C$ until needed.

2.2. Tissue preparation and cDNA library synthesis

Poly-A^+ mRNA was isolated from 60 adult female salivary glands (never blood fed, 1–5 days post-emergence) with the MicroFast track mRNA isolation kit (Invitrogen) following the manufacturer's protocol. The cDNA library was constructed in the pTriplEx2 vector using the SMART cDNA library construction kit (BD Clontech). Random clones were sequenced from the 5′ direction only, because successful sequencing from the 3′ end was usually lower than 40%. Full-length sequences were obtained in selected cases by performing primer-based extension protocols. See (Francischetti et al., 2002) for more details.

2.3. Gel electrophoresis and Edman degradation studies

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) of homogenized salivary glands of *Ae. albopictus* adult females was done using NuPAGE 4–12% bis-tris gel, 1 mm thick (Invitrogen). Salivary gland homogenates were treated with NuPAGE LDS sample buffer (Invitrogen) in the presence of β-mercaptoethanol (5%, v/v). Twenty pairs of homogenized salivary glands (~50 µg protein) were applied/lane. The gel was run with MES buffer according to the manufacturer's instructions. To estimate the molecular weight of the samples, See-Blue™ markers from Invitrogen (myosin, BSA, glutamic dehydrogenase, alcohol dehydrogenase, carbonic anhydrase, myoglobin, lysozyme, aprotinin, and insulin, chain B) were used. The gel was stained with Coomassie blue (0.2%, g/v). For aminoterminal sequencing of the salivary proteins, the gel was transferred to polyvinylidene difluoride (PVDF) membrane using 10 mM CAPS, pH 11.0, 10% methanol as the transfer buffer on a blot module for the XCell II Mini-Cell (Invitrogen). The membrane was stained with Coomassie blue (0.02% in the absence of acetic acid). Stained bands were cut from the PVDF membrane and subjected to Edman degradation using a Procise sequencer (Perkin-Elmer Corp.). More details can be obtained in a previous publication (Francischetti et al., 2002). To find the cDNA sequences corresponding to the amino acid (aa) sequence—obtained by Edman degradation of the proteins transferred to PVDF membranes from PAGE gels—we wrote a search program (in Visual Basic) that evaluated these aa sequences against the three possible protein translations of each cDNA sequence obtained in the mass sequencing project or against the full-length sequences derived in this work. For details, see (Valenzuela et al., 2002b).

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR) expression analysis

Total RNA was extracted from dissected glands, carcasses (adult females from which salivary glands had been dissected), and adult males using the TRIZOL reagent (Invitrogen) and treated with RNase-free DNase I.