MOLECULAR SURVEY FOR PATHOGENS AND MARKERS OF PERMETHRIN RESISTANCE IN HUMAN HEAD LICE (PHTHIRAPTERA: PEDICULIDAE) FROM MADAGASCAR

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KEY WORDS
Lice
Pediculus humanus capitis
Bartonella quintana
Acinetobacter
Acinetobacter baumannii
Permethrin Resistance
Madagascar

Human lice, Pediculus humanus, are host-specific blood-sucking obligate ectoparasites. Pediculus lice of humans consist of 2 genetically similar forms, the head louse, Pediculus humanus capitis De Geer 1767, and body louse, Pediculus humanus humanus L. 1758, that exhibit unique morphological, behavioral, and ecological characteristics, and functional adaptations (Durden and Musser, 1994; Veracx and Raoult, 2012). Human body lice are associated with poor sanitary conditions and typically persist in areas affected by famine, population displacement, and civil instability (Fournier et al., 2002; Cestari and Martignado, 2005; Ibarra et al., 2009). Body lice have been responsible for large outbreaks of epidemic typhus caused by Rickettsia prowazekii and trench fever caused by Bartonella quintana (Bechah et al., 2008; Badiaga and Brouqui, 2012; Bonilla et al., 2013). Body lice also vector Borrelia recurrentia, which causes louse-borne relapsing fever, currently endemic to East Africa, with the highest number of cases observed in Ethiopia (Badiaga and Brouqui, 2012). Furthermore, there are reports associating body lice with the spread of plague caused by Yersinia pestis (Drali et al., 2015; Dean et al., 2018). In the modern world, there are limited locations with the widespread endemic occurrence of body lice. However, they can be also found on homeless and other neglected populations in developed countries (Bonilla et al., 2009, 2014), and their increasing occurrence in those settings triggers public health concerns.

In contrast, human head lice have a cosmopolitan distribution, and it is estimated that they cause hundreds of millions of new infestations annually, mostly involving 3- to 14-year-old children (Mumcuoglu et al., 2006; Bonilla et al., 2013). Head-louse pediculosis is commonly perceived as an age-dependent rite of passage and an embarrassing social nuisance that can lead to social isolation and ostracism of the affected individuals (Pontius, 2014). Head-louse outbreaks do not raise the public health concerns that are associated with body lice because they are not considered to be effective vectors of known human disease-causing agents (Kim et al., 2011; Previte et al., 2014). Head-louse pediculosis is often responsible for uncontrolled exposure of children to various pediculicides and home remedies (Naehler et al., 2009). Synthetic pyrethroids, such as permethrin, are the most frequently used component of pediculicidal shampoos (Mazurek and Lee, 2000; Yoon et al., 2014). However, the majority of head lice from developing countries exhibit resistance to permethrin due to the spread of 3 knockdown resistance (kdr) point mutations (M81I, T92I, and L92F) in the voltage-sensitive sodium-channel alpha-subunit gene (Lee et al., 2003; Yoon et al., 2008; Drali et al., 2012; Durand et al., 2012). It has been suggested
that M815I and L923F are responsible for reduced susceptibility to permethrins, but the presence of the T929I mutation is the main cause of resistance (Drali et al., 2012). kdr-Type resistance has been reported in many countries and reaches 98.5% across multiple sampling sites in the United States (Durand et al., 2007; Drali et al., 2012; Toloza et al., 2014; Gellaty et al., 2016; Eremerova et al., 2017; Ponce-Garcia et al., 2017).

Several recent studies have reported the detection of deoxyribonucleic acid (DNA) of *B. quintana* and *Acinetobacter baumannii* in human head lice (Bonilla et al., 2009; Angelakis et al., 2011a, 2011b; Bouvresse et al., 2011; Kempf et al., 2012a; Diatta et al., 2014; Sunantaraporn et al., 2015; Eremerova et al., 2017; Mana et al., 2017; Candy et al., 2018; Louni et al., 2018a, 2018b). Besides being a known cause of nosocomial and community-acquired infections, *A. baumannii* is receiving increased attention because of the circulation of multidrug-resistant strains, including those detected in association with human head lice (Kempf et al., 2012b; Evellard et al., 2013; Antunes et al., 2014). These observations, coupled with additional experimental evidence of the vector competence of *P. h. capitis* and infrequent findings of DNA of other human and animal pathogens in lice, suggest that head lice may contribute more to the morbidity of community-acquired infections than is generally appreciated by health officials (Amanzougaghene et al., 2016, 2017; Kim et al. 2017).

Madagascar is an island nation in the Indian Ocean, off the coast of East Africa. A previous study on a small sample of head and body lice from Madagascar reported the detection of *B. quintana* in 2.7% (*n* = 75) and 4.5% (*n* = 22) of lice, respectively (Sangare et al., 2014). That study also linked increased occurrence of *B. quintana* and pediculosis with poor economic conditions in Africa (Sangare et al., 2014). The purpose of the present study was to conduct a molecular survey of head lice collected in 6 rural villages in southeastern Madagascar to better understand the role of lice as potential vectors of infectious disease agents in isolated communities living in poverty. Lice were examined for the occurrence of biomarkers of permethrin resistance and for evidence of infection with 2 louse-borne bacteria, *B. quintana* and *Acinetobacter* spp., including *A. baumannii*.

**MATERIALS AND METHODS**

**Sample collection**

Head lice (*n* = 159) were collected from 39 individuals from 6 rural villages in the southeastern tropical rain forests of Madagascar situated around Ranomafana National Park in the Ifanadiana district (21°02′–21°25′S, 47°18′–47°37′E) (Fig. 1). Villagers belonged to 2 cultural tribes, both of which practice social head-louse removal behaviors in which head lice are collected manually and then discarded. Discarded lice were voluntarily donated and stored in 1.6-ml polypropylene vials containing 70% ethanol, with each vial containing lice from 1 individual. Host gender and collection date were recorded for each vial. Individuals who donated lice gave verbal or written consent. All research protocols were approved by the Madagascar Secretary General and Direction Générale des Forêts (Permit #: 245/14/MEF/SG/DGF/DCB/SAP/SE). Approvals from village chiefs (Ampanjaka) and Ifanadiana district authorities were also obtained. The U.S. Veterinary Permit for Importation and Transportation of Controlled Materials and Organisms and Vectors (Permit #: 107234) was used for the importation of samples.

**Louse identification and DNA extraction**

Each louse specimen was examined under a dissecting microscope and identified to species, life stage, and sex using taxonomic keys (Tarasevich et al., 1988; Durden, 2004). Selected specimens were photographed using a Visionary digital K2/SC long-distance microscope (Infinity Photo-Optical Company, Boulder, Colorado), courtesy of Lorenza Beati (Georgia Southern University, Statesboro, Georgia). DNA extraction was performed separately for each louse using the DNeasy blood and tissue kit (Qiagen) with 10 U of *Ssp*I restriction enzyme (New England BioLabs, Beverly, Massachusetts) at 37 C. Digested fragments were visualized under ultraviolet (UV) light. Each amplicon was digested overnight with 10 U of *Ssp*I restriction enzyme (New England BioLabs) and the samples were stored at 4 C.

**Polymerase chain reaction (PCR) amplification and analysis of the kdr fragment**

The 332-base pair (bp) fragment of the 2-subunit of the sodium channel gene known to contain the resistant kdr mutation was amplified using end-point PCR (Durand et al., 2007; Eremerova et al., 2017). The PCR reaction was set up using 2X Taq PCR Master Mix kit (Qiagen), 2 µl of purified louse DNA, and 2 µl of 1 µM kdr forward and reverse primers (Table 1) for a total volume of 20 µl. The PCR cycling conditions were the same as described by Durand et al. (2007) and an Eppendorf Master Cycler (Eppendorf, Hamburg, Germany) was used. The PCR products were detected after electrophoretic separation in 1% agarose gels supplemented with ethidium bromide and visualized under ultraviolet (UV) light. Each amplicon was digested overnight with 10 U of *Ssp*I restriction enzyme (New England BioLabs, Beverly, Massachusetts) at 37 C. Digested fragments were
Table I. Primers and probes used in this study.

<table>
<thead>
<tr>
<th>Target organism</th>
<th>Target gene</th>
<th>Primer or probe name</th>
<th>Primers and probe sequence (5’–3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head lice</td>
<td>332-Base-pair portion of sodium channel a-subunit</td>
<td>kdr-F AAATCGTGGCCAACGTTAAA</td>
<td>Durand et al., 2007</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>kdr-R TGAATCCATTCACCGCATAA</td>
<td>Bouvresse et al., 2011</td>
<td></td>
</tr>
<tr>
<td>Bartonella quintana</td>
<td>3-Oxoacyl synthase</td>
<td>Fab3F GCTGGCCTTGCTCTTGATGA</td>
<td>Angelakis et al., 2011b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fab3R GCTACTCTGCGTGCCTTGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fab3 probe 6FAM-TGCAGCAGGTGGAGGAGAACGTG-TAMRA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acinetobacter species including A. baumannii</td>
<td>rpoB gene</td>
<td>rpoBF TACTCATATACCGAAAAGAAACGG</td>
<td>Bouvresse et al., 2011</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>rpoBR GGYTTACCAAGRCTATACTCAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>rpoB probe 6FAM-CGCGAAGATATCGGTCTSCAAGC-TAMRA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>OXA-51-like</td>
<td>OXA51-51-F 5’-TAATGCTTTGATCGGCCTTG-3’</td>
<td>Woodford et al., 2006; Mostachio et al., 2009</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>OXA51-51-R 5’-ATTTCTGACCGCATTTCCAT-3’</td>
<td>Mostachio et al., 2009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OXA-23-like</td>
<td>OXA23-23-F 5’-GATCGGATTGGAGAACCAGA-3’</td>
<td>Woodford et al., 2006; OXA23</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>OXA23-23-R 5’-ATTTCTGACCGCATTTCCAT-3’</td>
<td>Mostachio et al., 2009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OXA-24-like</td>
<td>OXA24-24-F 5’-GGTTAGTTGGCCCCCTTAAA-3’</td>
<td>OXA24</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>OXA24-24-R 5’-AGTTGAGCGAAAAGGGGATT-3’</td>
<td>Mostachio et al., 2009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IMP-like</td>
<td>IMP-F 5’-GAATAGAATGGTTAACTCTC-3’</td>
<td>Mendes et al., 2007; IMP</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Imp-R 5’-GAATAGAATGGTTAACTCTC-3’</td>
<td>Mostachio et al., 2009</td>
<td></td>
</tr>
</tbody>
</table>

DNA of *B. quintana* was detected using a species-specific TaqMan assay that amplified a fragment of the Fab3 gene (Angelakis et al., 2011b). The TaqMan assay reaction mixture was prepared using 10 μl of 2× Brilliant III Master Mix (Agilent Technologies, Santa Clara, California), 4 μM of both Fab3 forward and reverse primers, 8 μM of probe (Table I), and 5 μl of purified DNA. A cloned recombinant plasmid with the *B. quintana* Fab3 gene insert was used as a positive control, and DNase-free water was used as a negative control in each TaqMan run. The PCR cycling conditions consisted of an initial denaturation at 95 °C for 3 min, then 45 cycles of denaturation at 95 °C for 3 sec, hybridization and elongation at 55 °C for 30 sec. A CFX96 real-time system (Bio-Rad, Hercules, California) was used to perform all TaqMan reactions and postamplification analyses. Follow-up confirmation was performed by amplification and sequencing of the *Bartonella* RibC gene as described elsewhere (Wikswo et al., 2007).

Detection and identification of *Acinetobacter* DNA

A TaqMan assay targeting the specific fragment containing the RpoB gene of *Acinetobacter* species was performed using DNA extracted from adult lice according to a previously published protocol (Bouvresse et al., 2011; Ereemeeva et al., 2017). PCR reactions were set up using 10 μl of 2× Brilliant III Master Mix (Agilent Technologies), of 200 nmol of each 2 μM forward and reverse primer, 5 μl of 2 μM probe, and 4 μl of purified louse DNA. DNA of an environmental isolate of *A. baumannii* was used as a positive control and DNase-free water was used as a negative control in each TaqMan run. Each reaction consisted of 40 cycles of denaturation at 95 °C for 10 sec, primer annealing at 50 °C for 20 sec, and elongation at 68 °C for 30 sec. A CFX96 real-time system (Bio-Rad, Hercules, California) was used to perform all TaqMan reactions and postamplification analyses. Follow-up confirmation was performed by amplification and sequencing of the *Acinetobacter* species including *A. baumannii* of 200 nmol of each 2 μM of both Fab3 forward and reverse primers, 8 μM of probe (Table I), and 5 μl of purified DNA. A cloned recombinant plasmid with the *Fab3* gene insert was used as a positive control, and DNase-free water was used as a negative control in each TaqMan run. The PCR cycling conditions consisted of an initial denaturation at 95 °C for 3 min, then 45 cycles of denaturation at 95 °C for 3 sec, hybridization and elongation at 55 °C for 30 sec. A CFX96 real-time system (Bio-Rad, Hercules, California) was used to perform all TaqMan reactions and postamplification analyses. Follow-up confirmation was performed by amplification and sequencing of the *Acinetobacter* RibC gene as described elsewhere (Wikswo et al., 2007).

Detection of *A. baumannii* genes encoding for antibiotic resistance

DNA samples that tested positive by *rpoB* TaqMan were examined using end-point PCR to amplify the chromosomal *blaOXA1-like* gene followed by further analysis of all positive DNA samples using primers targeting *blaOXA23*, *blaOXA24*, *blaIMP*, and *blaVIM* (Table I). All PCR reactions were set up and performed according to previously described protocols (Mostachio et al., 2009) with an Eppendorf Master Cycler, and results were verified after electrophoresis of amplicons in 1% agarose ethidium bromide gels. Positive controls used for each test were kindly provided by J.-M. Rolain (Aix-Marseille Université, Marseille, France); sterile DNase- and RNase-free water were used as a nontemplate control for all reactions.

Sequencing and sequence analysis

PCR amplicons were gel purified using the Wizard SV gel and PCR cleanup system (Promega, Madison, Wisconsin). Sanger sequencing was performed using the corresponding forward and reverse primers for the corresponding gene by the Clemson
Table II. Frequency of the kdr-like T929I allele in human lice from Madagascar and prevalence of pathogens.

<table>
<thead>
<tr>
<th>Village</th>
<th>Female</th>
<th>Male</th>
<th>Nymphs</th>
<th>Total infestation</th>
<th>louse mean intensity</th>
<th>S/S</th>
<th>S/R</th>
<th>R/R</th>
<th>Co-infection, number (%)</th>
<th>Prevalence (95% CI)</th>
<th>Prevalence (95% CI)</th>
<th>Co-infection, number (%)</th>
<th>Prevalence (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ranomafana</td>
<td>34</td>
<td>2</td>
<td>2</td>
<td>39</td>
<td>2.2</td>
<td>63</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(1.7–10.5)</td>
<td>20/57 (1.7–10.5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tsararano</td>
<td>11</td>
<td>2</td>
<td>2</td>
<td>15</td>
<td>1.1</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(0.5–1.7)</td>
<td>0.42</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ambatolahy</td>
<td>38</td>
<td>6</td>
<td>6</td>
<td>51</td>
<td>6.3</td>
<td>34</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(2.6–30.1)</td>
<td>17.31</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ranovao</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>1.0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(0.2–0.6)</td>
<td>0.0962</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ambatovory</td>
<td>10</td>
<td>4</td>
<td>0</td>
<td>14</td>
<td>5.0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(1.0–3.5)</td>
<td>14.3%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mangevo</td>
<td>2</td>
<td>9</td>
<td>4</td>
<td>15</td>
<td>4.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(2.6–10.0)</td>
<td>0.556</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>39</td>
<td>159</td>
<td>99</td>
<td>202</td>
<td>4.1</td>
<td>99</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(2.6–10.0)</td>
<td>0.39</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* RFLP, restriction fragment length polymorphism; S, susceptible to permethrin or wild-allele; R, resistant or mutant allele.
† Denominator corresponds to the number of lice tested; numerator corresponds to the number of positive samples.
‡H, observed heterozygosity; E, expected heterozygosity; F, inbreeding coefficient, negative value indicates heterozygote excess.

**RESULTS**

**Head louse collections and characteristics**

Head louse samples (n = 159) were collected in 6 different rural villages in the southeastern rain forests of Madagascar in the Ifanadiana district (Fig. 1; Table II). The northern 3 sites (Ranomafana, Tsararano, Ambatolahy) and southern 3 sites (Mangevo, Ambatovory, Ranovao) are separated by a river. The inhabitants of these villages belong to 2 different cultural tribes, both of which practice social head-lice removal. The lice were collected from heads and necks from 39 individuals (n = 35 females, n = 4 males) ranging from 2 to 71 yr of age. The majority of samples were from Ranomafana village and Ambatolahy village, where they were collected from 21 and 6 individuals, respectively. Ambatolahy had a higher louse mean intensity per individual, 6.3, in comparison with 4.2 in Ranomafana, but these observations are not statistically significant (P = 0.8181).

Collected head lice comprised 45 males, 64 females, and 50 nymphs. Individual specimens were photographed and their morphological characters compared with head lice collected in Georgia (Fig. 2). Malagasy female head lice were darker and wider in the lower abdominal area, in contrast to female lice from Georgia, which were described in previous publications (Boniila et al., 2013; Ereemeeva et al., 2017). Malagasy male head lice had similar morphology and dimensions compared with male head lice from Georgia except they were darker and had thicker appendages. The female head louse from Madagascar shown in Figure 2B is also engorged with host blood, which has rendered it darker than other samples and also affected its overall abdominal size and shape. The only other prominent feature we observed in the Malagasy head lice was a tendency for slightly deeper
incrasations between the paratergal plates along the sides of the abdomen, although this character was variable between U.S. and Malagasy lice and could not be used to consistently distinguish between them. Also, because of the engorged state of the female head louse from Madagascar shown in Figure 2, these incrasasions are not as deep as in unfed females. Morphological characters that can often be used to separate human head and body lice (shape of the third antennal segment and extent of the paratergal plate apices extending into the intersegmental membranes) confirmed our samples as head lice (Tarasevich et al., 1988).

All available louse DNA samples were used for the detection of the 332-bp fragment of the α-subunit of the sodium-channel gene. All DNA samples were tested for *B. quintana*; however, only 107 adult lice were used in the detection of the *Acinetobacter* rpoB. Several lice were preserved for slide mounting and imaging.

**PCR and restriction fragment length polymorphism (RFLP) analysis of the kdr fragment**

To determine the occurrence and frequency of mutations linked to permethrin resistance, we performed SspI RFLP analysis of the *kdr* fragment PCR amplified from individual louse DNA samples. After *SspI* restriction enzyme digestion and separation by 2% agarose gel electrophoresis, 3 different profiles were observed (Fig. 3; Table II) and confirmed by sequencing. One type of RFLP pattern was detected in 47 (29.6%, 95% CI 23–37.1%) louse DNA samples that consisted of a single 322-bp fragment migrating as the original PCR amplicon, thus lacking a mutation and corresponding to the wild-type homozygous allele. A second RFLP pattern consisted of 2 fragments of 261 bp and 71 bp originating from an ATT|ATT *SspI* restriction site due to the T929I *kdr* mutation; it was detected in amplicons from 13 (8.2%, 95% CI 4.8–13.5%) louse DNA samples. The third RFLP profile, which represented the heterozygote genotype (322-, 261-, and 71-bp pattern), was predominant and detected in 99 (62%, 95% CI 54.5–69.4%) lice. Overall, the presence of the *kdr*-resistant allele was detected with an estimated frequency index of 0.39; it ranged between 0.28 and 0.5 among the 6 locations, but these variations were not significantly different (*P* = 0.06043). There were no significant differences in frequency of the T929I mutation between the northern and southern villages separated by the river (*P* = 0.61). The Hardy–Weinberg permutation test performed with 17,000 permutations indicated significant deficit of S/S and R/R homozygote genotypes and predominance of heterozygote S/R genotype (observed test statistic 14.78645 and *P* = 0.0001764706) and an inbreeding coefficient, *F* < 0. These estimates suggest that distribution of *kdr* genotypes detected in this louse population deviated significantly from Hardy–Weinberg equilibrium.

**Detection of louse-borne pathogens**

DNA from *B. quintana* was detected in 12.6% (95% CI 8.3–18.6%) of the 159 head lice tested (Table II). Specifically, *B. quintana* DNA was detected in lice that originated from 4 of the 6 villages. The prevalence ranged from 4.3% (95% CI 1.7–10.5%) in Ranomafana to 100% (95% CI 72.3–100%) in Ambatovory. The average prevalence of 3.6% *B. quintana* detected in head lice from northern villages (Ranomafana, Tsararano, and Ambatolaha) was significantly lower compared with the 71.4% prevalence of *B. quintana* detection in head lice from the 3 southern sites (*P* < 0.05). Detection of *B. quintana* DNA was further confirmed by amplification and sequencing a RibC gene fragment (NCBI accession numbers MK409744 and MK409745).

Because of the limited quantity of DNA available from nymphs, only a subset of 107 adult samples was tested for DNA of *Acinetobacter* spp. using the TaqMan assay (Table II). DNA of *Acinetobacter* spp. was detected in 42.1% (95% CI 33.1–51.5%) of the 107 DNA extracts from adult head lice. Louse samples positive for *Acinetobacter* spp. DNA were found in all villages; its prevalence ranged between 20% (95% CI 3.6–62.5%) in Mangevo and 100% in both Tsararano (95% CI 34–100%) and Ranovo (95% CI 34–100%). Sequencing performed for several amplicons identified only *A. baumannii* (NCBI accession number MH801987, MH801998). There were at least 2 genetic types of *A.
baumannii rpoB differing in an A/G single-nucleotide polymorphism in nucleotide position 96 of the amplicon.

DNA from both Acinetobacter spp. and B. quintana was simultaneously detected in 3.5% (n = 57, 95% CI 1–12%) of the head lice from Ranomafana and 3% (n = 10, 95% CI 10.8–60.3%) of the head lice from Ambatovory. Furthermore, DNA from Acinetobacter was also found in 5 lice with the kdr resistance allele. These infected and potentially permethrin-resistant lice were from Ranomafana (1 of 8), Ambatolahy (2 of 3), and 1 in each from Ranovao and Mangevo.

Detection of antibiotic resistance genes in Acinetobacter

Detection of antibiotic resistance genes was performed using 24 DNA extracts from head lice that tested positive for Acinetobacter rpoB TaqMan with cycle threshold values ≤30. Fourteen of the 24 lice tested positive for the blaOXA-1-like gene fragment using endpoint PCR and thus were identified as A. baumannii. All 14 blaOXA-1-like gene-positive lice tested negative for blaOXA-23, blaOXA-24, blaLB, and blavIM.

DISCUSSION

In this study, we performed molecular surveys of head lice infesting Malagasy human populations in Madagascar. We determined the presence of the kdr resistance allele and of B. quintana and Acinetobacter spp. including A. baumannii in these ectoparasites.

Madagascar belongs to a group of the least-developed countries. The economy of the country was heavily affected by the political crisis during 2009–2013, resulting in low quality of life for the majority of the Malagasy population (Barmania, 2015). Many Malagasy have difficulty with access to health care, particularly individuals living in impoverished rural communities, which may have more barriers to good hygiene practices (Dell et al., 2012).

Madagascar is endemic for many traditional tropical diseases, including malaria; however, emergence and re-emergence of other vector-borne diseases such as plague have further affected the well-being of the nation in recent years (D’Ortenzio et al., 2018). Despite the high prevalence of vector-borne diseases, Madagascar also has the highest frequency use of insecticide-treated nets in Africa. Coupled with residual indoor spraying, this is credited with generating the lowest malaria prevalence in tropical Africa (Kesteman et al., 2014). However, the broad application of insecticides may also be a leading cause in the development of insecticide resistance in mosquitoes and other blood-sucking arthropods. As an example, the Oriental rat flea, Xenopsylla cheopis, tested from different correctional facilities in Madagascar, exhibited various patterns of susceptibility and resistance to different insecticides including permethrin (Miarinjara et al., 2017).

We determined whether any distinctive morphological features could be identified in Malagasy head lice compared with head lice collected in the United States and other locations (Tarasev et al., 1988; Durden 2004). Head lice from Madagascar were consistently darker than head lice from the United States (Fig. 2). Ewing (1926) and Veracx et al. (2012) have similarly noted that some populations of head lice from Africa are more darkly pigmented than head lice from North America or Europe.

Sangare et al. (2014) also reported that head lice from Madagascar, Senegal, and Ethiopia are black in color, whereas African body lice varied from black to brown and gray depending on their country of origin (Sangare et al., 2014). It could be argued that the darker color of some African head lice is adaptive for better camouflage next to darker skin; however, we did not have samples from Africans of other ethnic groups to verify this speculation. On the other hand, it should be mentioned that the Malagasy population largely originated from Southeast Asia (Indonesia–Java) (Cox et al., 2012), and individuals in Ranomafana, in particular, have much more Southeast Asian phenotype than African.

Human head lice from rural Madagascar were tested for 1 of the 3 markers of permethrin resistance, specifically the T929I kdr mutation. This mutation was detected in 70% of lice from all 6 villages sampled; 62% of positive lice were heterozygous and 13% were homozygous for this marker. Overrepresentation of the heterozygotes in this louse population is suggestive of active selective pressure conferred by advantages associated with heterozygosity, as has been proposed in other louse surveillance studies (Thomas et al., 2006). This has also been observed with some other arthropods (Lira et al., 2016; Aguilar-Tipamam et al., 2015; Platt et al., 2015; Viana-Medeiros et al., 2017). Heterozygous ladybird beetle Eriopis connexa (Germar) (Coleoptera: Coccinellidae) resistant to lambda-cyhalothrin have fitness advantages including greater fecundity and longevity compared with the homozygous resistant population (Lira et al., 2016).

Inheritance of pyrethroid resistance and the sodium-channel mutation in Rhipicephalus (formerly Boophilus) microplus also provides survival advantages; however, the phenotype of heterozygotes and resistance pattern to various pyrethroids depended on maternal or paternal source of the resistant kdr allele (Aguilar-Tipamam et al., 2008). Anopheles gambiae kdr heterozygote male mosquitoes from Burkina Faso were more likely to mate than homozygote resistant males (Platt et al., 2015). kdr-Resistant homozygotes were found in 32% and 43% of 2 populations of Brazilian Stegomyia aegypti (=Aedes aegypti); however, even at this level mating effectiveness was equally impaired and females from the second group exhibited a lower rate of blood feeding and lower fecundity (Viana-Medeiros et al., 2017).

The T929I kdr mutation is 1 of 3 kdr mutations (M815I-T917I-L920F) found in human head lice from the United States, Canada, France, Argentina, and Mexico where the kdr-resistant allele was detected in up to 98.3% of lice tested (Tolozha et al., 2014; Yoon et al., 2014; Eremeeva et al., 2017; Ponce-Garcia et al., 2017). Many of these studies reported the prevalence of kdr homozygote resistant lice (Tolozha et al., 2014; Yoon et al., 2014; Eremeeva et al., 2017). The appearance and broad spread of these knockdown mutations in human lice in developed countries have been associated with uncontrolled use of over-the-counter pyrethroid-based pediculicides (Yoon et al., 2014). It has not been associated with any detrimental effects or fitness disadvantages in U.S. head-louse populations (Yoon et al., 2014; Gellaty et al., 2016). Since pediculicides are not used in rural Madagascar, it is possible that broad environmental application of pesticides is responsible for the occurrence of mutated kdr alleles in human Malagasy lice similar to its occurrence in mosquitoes and fleas (Miarinjara et al., 2017; Rakotoson et al., 2017). Further testing using live lice would be required to determine their susceptibility to pesticides using bioassays and analysis of kdr-resistant
inheritance and its phenotypic manifestations as fitness benefits in various louse populations.

Our study corroborates previous findings of B. quintana in head and body lice in Madagascar (Sangare et al., 2014), and demonstrates its presence in another geographic location on the island. In our study, the prevalence of B. quintana DNA was 12.6% of 159 head lice, which is significantly higher than the 2.7% prevalence previously reported in 75 head lice collected in a 3-yr study (Sangare et al., 2014). The role of head lice in the maintenance of B. quintana remains unclear despite findings of Bartonella DNA in human head lice in different geographic locations (Bonilla et al., 2009; Angelakis et al., 2011a; Sangare et al., 2014). Overall, 2% of 616 head lice from 9 African countries tested PCR positive for B. quintana, ranging from 0 to 72.9% in lice from general populations and peaking to 89.7% and 93.9% in lice collected from individuals from jails and refugee camps, respectively (Sangare et al., 2014). Head lice collected from homeless individuals in the United States and the Netherlands had one of the highest prevalences of B. quintana DNA reported, 33.3% and 36%, respectively, although when comparing these observations, it should be considered that different molecular assays were used in the individual laboratories (Fournier et al., 2002; Bonilla et al., 2009). Body lice were not collected as a part of our study and were not observed on individuals from whom head lice were obtained. However, the co-occurrence of 2 human louse biotypes quite often occurs in African populations, and an average of 53.7% of 424 body lice from 9 African countries have tested positive for B. quintana (Sangare et al., 2014). Although body lice were not present in the Madagascar samples tested in this study, they can occur in these populations, according to some anecdotal reports. Communities affected by head and body lice should be vigilant about future outbreaks of febrile illnesses of unknown origin, keeping in mind that B. quintana is responsible for a variety of clinical conditions ranging from classic trench fever to bacillary angiomatosis and other symptoms and asymptomatic conditions (Foucault et al., 2006). Detection of Acinetobacter spp. DNA in 42.1% of 107 adult head lice tested from Madagascar was an important observation in our study. A subset of these samples tested positive for the A. baumannii blaoxa1-like gene, suggesting an overall estimated prevalence of this pathogen of 58% in human Malagasy lice that tested positive for Acinetobacter spp. rpoB (Kempf et al., 2012b). In comparison, the constitutive blaoxa1-like gene of A. baumannii was detected in 4% of human head lice from Senegal (Kempf et al., 2012b). Species of the A. baumannii complex are typically linked to outbreaks of nosocomial and community-acquired infections and cause wound and bloodstream infections, ventilator-associated pneumonia, and nosocomial meningitis in critically ill individuals (Eveillard et al., 2013). Recently, human lice have been increasingly regarded as an emerging source of human exposure to Acinetobacter spp., a concern supported by its detection in or isolation from both head lice and body lice, respectively (La Scola and Raoult, 2004). Various studies have reported the detection of DNA of Acinetobacter spp. in human lice ranging from 3.0% to as high as 71% in different countries (La Scola and Raoult, 2004; Kempf et al., 2012b; Sunantaraporn et al., 2015), and the occurrence of several species of Acinetobacter has been reported (Sunantaraporn et al., 2015; Mana et al., 2017; Candy et al., 2018). As an example, Acinetobacter spp. DNA was detected in 27% (n = 67) of head lice, and identified as A. baumannii (14%), Acinetobacter johnsonii (11%), and Acinetobacter variabilis (2%) (Mana et al., 2017). In France, Acinetobacter DNA was detected in 11.5% of head lice (Candy et al., 2018). Acinetobacter baumannii was the most prevalent species, but Acinetobacter calcoaceticus, Acinetobacter nosocomialis, Acinetobacter junii, and 2 new species Candidatus Acinetobacter Bobigny-1 and Bobigny-2 were also identified in these head lice (Candy et al., 2018). Acinetobacter DNA was also detected in 54.1% (n = 37) of head lice collected from Nigerian refugees, and in 55.6% (n = 45) of head lice collected from Algerian school children (Louni et al., 2018a). All Nigerian head lice that were positive for Acinetobacter spp. had A. baumannii, whereas DNA of A. johnsonii (60%), A. variabilis (32%) and A. baumannii (8%) was identified in head lice collected from Algerian schoolchildren (Louni et al., 2018a).

In conclusion, our study provides molecular data suggesting a high prevalence of human pathogens in human head lice infesting individuals living in poor and overcrowded communities in Madagascar. These observations, together with the detection of a high prevalence of kdr mutations in these lice, indicate that they pose a significant public health risk. Further biological assessments are needed to determine the susceptibility of these lice to nonpermethrin pediculicides and other control remedies. Further studies are also warranted to address local barriers to the elimination and control of human lice.

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LITERATURE CITED


Tarasevich, I. V., A. A. Zemskaya, and V. V. Khudobin. 1988. Diagnosis of the species of lice in the genus Pediculus. Meditsinskaia Parazitologiia i Parazitarnye Boleznii 3: 48–52. [In Russian]


