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Determination of knockdown resistance allele frequencies in global human head louse populations using the serial invasive signal amplification reaction

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Abstract

BACKGROUND—Pediculosis is the most prevalent parasitic infestation of humans. Resistance to pyrethrin- and pyrethroid-based pediculicides is due to knockdown (*kdr*)-type point mutations in the voltage-sensitive sodium channel α-subunit gene. Early detection of resistance is crucial for the selection of effective management strategies.

RESULTS—*Kdr* allele frequencies of lice from 14 countries were determined using serial invasive signal amplification reaction. Lice collected from Uruguay, UK and Australia had *kdr* allele frequencies of 100% while lice from Ecuador, Papua New Guinea, South Korea and Thailand had *kdr* allele frequencies of 0%. The remaining 7 countries investigated, including seven US populations, two Argentina, Brazil, Denmark, Czech Republic, Egypt and Israel, displayed variable *kdr* allele frequencies, ranging from 11% to 97%.

CONCLUSION—The newly developed and validated SISAR method is suitable for accurate monitoring of *kdr* allele frequencies in head lice. Proactive management is needed where *kdr*-type resistance is not yet saturated. Based on sodium channel insensitivity and its occurrence in louse populations resistant to pyrethrin- and pyrethroid-based pediculicides, the T917I mutation appears a key marker for resistance. Results from the Egyptian population, however, indicate that phenotypic resistance of lice with single or double mutations (M815I and/or L920F) should also be determined.

Keywords

genotyping; human head lice; *kdr* allele frequency; *Pediculus humanus capitis*; pyrethroid resistance; SISAR

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1 INTRODUCTION

The human head louse, *Pediculus humanus capitis* De Geer (Anoplura: Pediculidae), is an obligatory hematophagous ectoparasite that causes prevalent infestations of humans (pediculosis) in the United States (US) and other countries[1] despite the availability of many over-the-counter (OTC) pediculicidal products. Over the last two decades, many cases of resistance to pyrethrin- and pyrethroid-based pediculicides have been documented.[2-14] Knockdown resistance (*kdr*) is a major attribute of resistant lice and supports the claim that treatment failures are largely due to insecticide resistance.[14] This heritable *kdr* trait is associated with nerve insensitivity to DDT, the pyrethrins and the pyrethroids, which was first identified in the house fly, *Musca domestica* L.[15,16] Three point mutations, resulting in the amino acid substitutions M815I, T917I and L920F in the *para*-orthologous voltage-sensitive sodium channel (VSSC) α-subunit, have been identified in permethrin- and DDT-resistant head lice.[14, 17] These mutations result in permethrin insensitivity in house fly Vssc1 channel variants when heterologously expressed in *Xenopus laevis* (Daudin) oocytes and examined electrophysiologically using two-electrode voltage clamp techniques.[18]

The *kdr*-type resistance in head louse populations appears to be widespread, but varies in intensity and is not yet uniform.[11] Thus, the establishment of a proactive resistance management strategy is essential to maximize the time over which the pyrethrin- and pyrethroid-based pediculicides remain effective control agents. Detection of resistance when the frequency of resistant lice is still low is crucial for implementing proper management decisions that can delay and reverse resistance development. Early resistance detection, however, is often difficult using conventional mortality bioassay-based monitoring methods, particularly when the resistance trait is recessive. Several genotyping techniques for the determination of *kdr* allele frequencies using genomic DNA extracted from individual head lice have been developed to overcome this limitation and to improve time and cost effectiveness.[19-23]

One such technique, serial invasive signal amplification reaction (SISAR), employs a fluorescence resonance energy transfer (FRET) detection format[24] and has been used for the high-throughput detection of the *kdr* mutations (T917I and L920F mutations) in field populations of head lice.[19] The SISAR method uses PCR-amplified DNA targets prepared from individual lice and has been found to be a very accurate and efficient technique in obtaining detailed information on the genotypes and *kdr* allele frequencies of human head louse populations.[23]

In this investigation, we report the development and validation of a M815I mutation kit using the SISAR technology. Using this new kit and two previously developed kits, we provide data of *kdr* allele frequencies at all three mutation sites in head louse populations collected from 14 different countries.

2 MATERIALS AND METHODS

2.1 Head louse populations

Head lice were collected from 14 countries by volunteers (Table 1). Each population consisted of 8-158 lice, and a total of 343 lice were used for SISAR analyses. Information on number of infested humans who willingly provided the collected lice for SISAR analyses was not disclosed in some collections, as indicated (Table 1), due to the human subject use protocols. Lice were stored in 70% ethanol at the collection sites and sent to the Environmental and Molecular Toxicology Laboratory, University of Massachusetts-Amherst for SISAR analyses.

2.2 Genomic DNA extraction and amplification of a 1.1 kb-length PCR fragment

Genomic DNA (gDNA) was extracted from individual head lice using DNAzol (MRC Inc., Cincinnati, OH) containing 1% (v/v) polyacryl carrier (MRC Inc.) following the manufacturer's instruction with a slight modification to handle small samples. A single louse was homogenized in 50 μL DNAzol using a 0.2 mL glass-glass homogenizer (Kontes Glass Co., Vineland, NJ) and the resulting homogenate transferred to a 0.5 mL microcentrifuge tube. The homogenizer was rinsed with 50 μL DNAzol that was transferred to the tube containing the homogenate. The tube was incubated at room temperature for 10 min and centrifuged at 10,000 × *g* for 10 min at room temperature. The supernatant was transferred to a new tube and mixed with an equal volume of 100% ethanol. Louse gDNA was precipitated at −20 °C for 30 min and pelleted by 10,000 × *g* centrifugation at 4 °C for 7 min. After removing the supernatant by pipetting, gDNA was washed with 70% ethanol (500 μL) and pelleted by 10,000 × *g* centrifugation at 4 °C for 1-2 min. The louse gDNA pellet was air dried for 5 min and dissolved in 10 μL nuclease-free water.

A 1.1 kb length fragment of the head louse VSSC α-subunit gene, encompassing the M815I mutation site as well as the previously established T917I and L920F mutation sites[14,19] was amplified using Advantage 2 Polymerase (Clontech, Mountain View, CA), 20-100 ng gDNA and specific primers (Forward: TGTGGCCTTACTTGTATTCGAC and Reverse: CATTGTCAGCGGTGGGAGCAGA) in a total reaction volume of 20 μL. The following thermal cycle program was used for PCR amplification: DNA denaturation at 95 °C for 1 min followed by 30 cycles of 95 °C for 30 s, 68 °C for 70 s (a combined step for annealing and extension), followed by a final extension at 68 °C for 10 min. All amplifications were carried out using the PCR Express thermal cycler, model HBPXBG02 (Hybaid Ltd., UK). Amplification of the fragment was confirmed by 8 g L^{-1} agarose gel electrophoresis using ethidium bromide staining. The PCR-amplified DNA fragment was purified using Microcon filters (Montage® PCR Centrifugal Filter Devices, Millipore, Billerica, MA) following manufacturer's instructions and the concentration of the DNA fragment determined with PicoGreen dsDNA quantification kit (Invitrogen, Carlsbad, CA). The DNA fragment was diluted with nuclease-free water to a final concentration of ~27 μg L^{-1} for optimization of SISAR conditions.

2.3 Development of a SISAR kit for detection of the M815I mutation

Based on the DNA sequence from the VSSC α-subunit gene from the head louse, a new SISAR genotyping kit for detection of the M815I mutation (AT**G** to AT**T**) was developed in conjunction with Third Wave Technologies-Agbio (Madison, WI).[17, 19] The invasive oligonucleotide and primary probes (G probe and T probe) were synthesized to form a trimeric structure with the PCR-amplified 1.1 kb DNA fragment (1.1 kb DNA target) in the reaction mixture (Fig. 1). The two synthesized 59 bp oligonucleotide targets with either the G or T nucleotides present (equivalent to susceptible or resistance genotypes, respectively) were provided by the manufacturer for control reactions and their sequences are underlined in the fragment of the 1.1 kb DNA target sequences of the head louse given in Fig. 1.

2.4 Optimization of SISAR conditions for the detection of the M815I, T917I, and L920F mutations

The 1.1 kb DNA targets were PCR-amplified using gDNA of individual head lice from Mathis, Texas (TMS-HL) as described in Section 2.2 and were fully sequenced to determine presence or absence of the three point mutations. The validated 1.1 kb DNA targets were subsequently used as standards to optimize the reaction conditions of the M815I, T917I, and L920F SISAR kits with respect to DNA concentration, reaction time, and incubation temperature previously described[19] with the following alterations. Fluorescence for the susceptible G and resistant T nucleotides in the M815I kit were determined using Redmond

Red (λ_{Ex} = 560 nm and λ_{Em} = 620 nm) and FAM (λ_{Ex} = 485 nm and λ_{Em} = 530 nm), respectively. Fluorescence for the susceptible C and resistant T nucleotides in the T917I and L920F kits were determined using FAM and Redmond Red, respectively. Net fold-over-zero (Net-FOZ) for each dye [Eq. (1); RFU : relative fluorescent units] and SISAR ratio [Eq. (2)] were determined according to the manufacturer's instructions for each SISAR kit:

Eq. (1) Calculation of Net-FOZ:

$$\text{M815I, T917I, and L920F kits: Net}-\text{FOZ T} = [(\text{RFU T probe}) / (\text{RFU with no target control})] - 1$$
$$\text{M815I kit: Net}-\text{FOZ G} = [(\text{RFU G probe}) / (\text{RFU with no target control})] - 1$$
$$\text{T917I and L920F kits: Net}-\text{FOZ C} = [(\text{RFU C probe}) / (\text{RFU with no target control})] - 1$$

Eq. (2) Calculation of SISAR Ratio:

$$\text{M815I kit:SISAR Ratio=Net}-\text{FOZ T/Net}-\text{FOZ G}$$
$$\text{T917I and L920F kits:SISAR Ratio=Net}-\text{FOZ T/Net}-\text{FOZ C}$$

2.5 SISAR genotyping of the M815I, T917I, and L920F mutations

PCR-amplified 1.1 kb DNA targets (~1-12 mg L^{-1}) were diluted to give a concentration of 0.2 ng DNA in 7.5 μL nuclease-free water, heat denatured at 95 °C for 10 min, and immediately placed on ice. For control reactions, 7.5 μL of susceptible and resistant oligonucleotide targets (25 mg L^{-1}) were used. The 1.1 kb DNA targets and oligonucleotide targets were transferred to individual wells of a 96 well plate (Greiner Bio-One, Frickenhausen, Germany). 7.5 μL of SISAR reagents (3 μL SISAR probe mix, 3.5 μL Cleavase XI FRET mix and 1 μL Cleavase XI enzyme) were aliquoted to each well containing either the 1.1 kb DNA targets or the oligonucleotide targets, and the contents of each well overlaid with 15 μL mineral oil. The plate was sealed with Microseal 'A' Film (MJ Research Inc., Waltham, MA) and incubated at 63.5 °C for 30 min, 66.5 °C for 1 h, and 63.5 °C for 1 h for SISAR genotypings of the M815I, T917I, and L920F mutations, respectively, in a Hybaid PCR Express Thermal Cycler. The RFU from each SISAR sample were measured using a Gemini XS fluorescent spectrophotometer (Molecular Devices Corp., Sunnyvale, CA) and net-FOZ values for each fluorescent dye and SISAR ratio determined as described in Section 2.4.

3 RESULTS

3.1 PCR-amplification of 1.1 kb DNA targets and gDNA sequencings

PCR-amplified 1.1 kb DNA targets, spanning the three point mutation sites (M815I, T917I and L920F mutations), were successfully obtained from all 27 TMS-HL gDNA samples (Fig. 2A). Each of these amplified DNA fragments was subsequently sequenced and used for optimization of the SISAR genotyping protocols below. During this process, 4 introns (86, 91, 86 and 98 bp) were identified (Fig. 2B).

3.2 Optimization of SISAR conditions for M815I, T917 and L920F mutations using TMS-HL

Standard reaction conditions for genotyping individual TMS-HL lice using SISAR were optimized with respect to DNA concentration, reaction temperature, and reaction time using the PCR-amplified 1.1 kb DNA targets, which spanned the M815I, T917I and L920F mutation sites. Actual genomic DNA sequences were validated by direct sequencing as being either resistant homozygote (R), heterozygote (H) or susceptible homozygote (S) at each mutation site (Table 2).

Regardless of reaction temperature and time (with the exception of the 15 min reaction time at both temperatures), the high concentration (20 ng reaction^{-1}) of 1.1 kb DNA target did not clearly allow discrimination between the homozygote and heterozygote genotypes using M815I SISAR kit (Fig. 3A). The low concentration (0.2 ng reaction^{-1}) of 1.1 kb DNA target, however, effectively allowed discrimination amongst the genotypes at both reaction temperatures and at all reaction times. Longer reaction times did not improve the discrimination amongst genotypes, although these improved the discrimination for the 59 bp oligonucleotiede targets (Fig. 3A).

Overall, a 30 min reaction time at 63.5 °C with 0.2 ng 1.1 kb DNA target/reaction was determined to yield the best discrimination amongst M815I genotypes (R, H, and S) (Fig. 3A). Following optimization, a SISAR ratio of 2.5 or greater was determined to be diagnostic of a louse that had the T nucleotides at both alleles (resistant homozygote, R) at the M815I site. A SISAR ratio of 0.25 or lower was determined to be diagnostic of a louse that had the G nucleotides at both alleles (susceptible homozygote, S) at the M815 site. A SISAR ratio of greater than 0.5 but lower than 2.0 was determined to be diagnostic of a heterozygous louse at the M815I site (T and G nucleotides present in equal quantities, H) (Fig. 2). Using these conditions, the M815I SISAR kit unambiguously identified the three genotypes, allowing for the estimation of *kdr* allele frequencies at each mutation site and ultimately for the construction of a global *kdr* map.

Similar to the M815I SISAR, the high concentration of the 1.1 kb DNA target (20 ng reaction^{-1}) failed to discriminate the three genotypes regardless of reaction temperatures or times using either T917I- or L920F-SISAR kits (Fig. 3B and 3C, respectively). The lower DNA concentration (0.2 ng reaction^{-1}) allowed discrimination amongst genotypes with both the T917I- and L920F-SISAR kits at a reaction time of 1 h and at 63.5 and 66.5 °C temperatures, respectively. Increasing the reaction time of the T917I-SISAR kit did not substantially improve the discrimination amongst genotypes at either reaction temperatures (Fig. 3B). Increasing the reaction time of the L920F-SISAR kit reduced discrimination amongst genotypes at the 66.5 °C reaction temperature and substantial discrimination between the olignucleotide targets was not observed until 2 h (Fig. 3C). Increasing reaction time at the 66.5 °C temperature reduced discrimination amongst genotypes, although substantial discrimination was still observed with the oligonuceotide targets.

Overall, a 1 h reaction time resulted in the effective genotyping of the 1.1 kb DNA target samples at the 0.2 ng reaction^{-1} concentration at either reaction temperature for both the T917I- and L920F-SISAR kits (Fig. 3B and 3C). Optimal SISAR conditions for the T917I mutation site were determined to occur at 1 h at 66.5 °C using 0.2 ng reaction^{-1} of the 1.1 kb DNA target (Fig. 3B). Optimal SISAR conditions for the L920F mutation site were determined to occur at 1 h at 63.5 °C using 0.2 ng reaction^{-1} of the 1.1 kb DNA target (Fig. 3C).

Consistent with the optimization of M815I SISAR kit, a SISAR ratio with the T917I and L920F kits for the resistant homozygote, R (T nucleotides at both alleles) was established at a value greater than or equal to 2.5 while the SISAR ratio for the susceptible homozygote, S (C nucleotides in both alleles) was established at a value less than or equal to 0.25. A SISAR ratio greater than 0.5 and less than 2 was designated as a heterozygote (T and C nucleotides were present in equal quantities, H) (Fig. 2).

The genotypes of the 27 individual TMS-HL samples were experimentally predicted using the optimized SISAR protocols and compared to those that were determined by automated sequencing (Table 2). These findings were consistent with those previously reported for the T917I and L920F mutation sites using SISAR genotyping.[19] Collectively, our findings

indicate that all three SISAR kits discriminated genotypes with 100% accuracy using a PCR-amplified 1.1 kb DNA target prepared from TMS-HL.

3.3 Diplotype determination

According to the genotypes at the three mutation sites, a diplotype of each louse was determined. For example, a TMS-HL louse (sample 1) had homozygous mutations at all three mutations sites (R genotype at M815I site, R genotype at T917I site, and R genotype at L920F site) (Table 2). This louse was determined to have a RRR diplotype.

The TMS-HL population was determined to comprise three different diplotypes (RRR, HHH and SSS). Three lice were resistant homozygotes (resistance diplotype, RRR), 14 lice were heterozygotes (heterozygous diplotype, HHH), and ten lice were susceptible homozygotes (susceptible diplotype, SSS) at all three point mutation sites (M815I, T917I, and L920F mutation sites, respectively).

3.4 Determination of *kdr* allele frequencies at the three mutation sites in human head louse populations collected globally

Each of the three kits produced SISAR ratios (three SISAR ratios/louse sample) that fell into the previously determined ranges and allowed the genotyping of individual lice at each mutation site collected globally. The *kdr* allele frequencies at the three mutation sites were determined for head louse populations collected from 14 countries using all three SISAR kits [Eq. (3)] and a global *kdr* map constructed (Fig. 4).

Eq. (3)

$$kdr \text{ allele frequency } (\%) = [\text{number of } kdr \text{ alleles in } n \text{ lice at the three mutation sites}/(n \text{ lice} \times 6)] \times 100, \quad \text{where } n = \text{num}$$

[c/e, *N* in Eq 3 has been replaced by *n*, as used for populations in the following paragraphs; please ask authors to confirm that this is OK. Tech. ed.]

All seven North American head louse populations were collected from the US (Table 1) and their diplotypes were determined by SISAR as follows, Pinon, Arizona (50% RRR, 30% HHH, 20% SSS); Ocklawaha, Florida (100% RRR); West Palm Beach, Florida (100% RRR); Asginaw, Michigan (96.6% RRR, 3.4 % HHH); Tracy, Minnesota (100% RRR); Mathis, Texas (11% RRR, 52% HHH, 37% SSS) and San Antonio, Texas (100% RRR). The combined data resulted in 63% RRR, 22% HHH and 15% SSS. Based on this result, *kdr* allele frequencies at the three mutation sites in the U.S. populations were 65 % (Pinon, Arizona), 100% (Ocklawaha, Florida), 100% (West Palm Beach, Florida), 98.3% (Saginaw, Michigan), 100% (Tracy, Minnesota) and 100% (San Antonio, Texas). The only US population that displayed *kdr* allele frequency less than 50% was Mathis, Texas (37%). The combined data from US populations resulted in a 74% *kdr* allele frequency.

In South America, five head louse populations were collected (Table 1). Head louse populations from Buenos Aires, Argentina (n = 54), Comodoro Rivadavia, Argentina (n =5) and Brazil (n = 12) had the diplotypes of 90.7% RRR, 5.6% HHH, 3.7% SSS; 60% RRR, 20% HHH, 20% SSS; and 50 %RRR, 25% HHH, 25% SSS, respectively. The Ecuador population (n=8) had 100% SSS and the Uruguay population (n = 8) had 100% RRR. The calculated *kdr* allele frequencies were 93.5% (Buenos Aires, Argentina), 70% (Comodoro Rivadavia, Argentina), 62.5% (Brazil), 0% (Ecuador), and 100% (Uruguay). The overall *kdr* allele frequency for South America was calculated to be 79.9%.

The *kdr* allele frequencies of louse populations collected from European Union (EU) were also varied, based on head lice collected from the UK (*n* = 8), Denmark (*n* = 12) and the Czech Republic (*n* = 7), where the diplotypes were 100% RRR in the U.K., 75% RRR, 17% HHH, 8% SSS in Denmark, and 29% RRR, 14% HHH, and 57% SSS in Czech Republic. Based on their diplotypes, *kdr* allele frequencies were 100% (Bristol, U.K.), 83.5% (Uvelse, Denmark), and 36% (Czech Republic). The overall *kdr* allele frequency for the EU was calculated to be 75.9%.

Only four head louse samples from Israel were analyzed in this study. No susceptible homozygote lice were identified. Diplotypes of the Israel lice were 75% RRR and 25% HHH, resulting in a 87.5% *kdr* allele frequency.

All head lice collected from Seoul and Hong-Sung, South Korea (*n* = 36), Thailand (*n* = 33), and Papua New Guinea (*n* = 3) possessed SSS diplotypes, resulting in 0% *kdr* allele frequencies. Comparatively, all head lice (*n* = 28) collected from Australia possessed the RRR diplotype, resulting in a 100% *kdr* allele frequency.

Interestingly, six different diplotypes were identified in lice (*n* = 20) from Kafr-el Sheikh Governorate, Egypt (Figs. 4 and 5). Fifty percent of the analyzed lice were HSH, 30% were RSR, and the remaining 4 lice possessed diplotypes of HSR, RHR, RSH, and HHH, respectively. Based on these results, the calculated *kdr* allele frequency for the Egyptian population was 47.5%. Unlike individual lice from other populations, however, no lice had either the RRR or SSS diplotype. This population had a 70% *kdr* allele frequency at the M815I mutation site, a 5% *kdr* allele frequency at the T917I mutation site, and a 70% *kdr* allele frequency at the L920F mutation site.

4 DISCUSSION

Early detection of head louse resistance to pyrethroid insecticides is a crucial factor for long-term resistance management designed to effectively suppress or slow the spread of this resistant pest.[19] As an alternative to conventional mortality bioassay-based monitoring, SISAR kits have been developed to estimate the diplotypes and the *kdr* allele frequencies of field-collected head louse populations. This technique, which allows for the determination of zygosity at each mutation site, is particularly useful to understand the population dynamics of *kdr* during the early stage of resistance because heterozygous lice can be detected in a population.

Developing a new SISAR kit for the detection of M815I mutation and using a PCR-amplified 1.1 kb DNA target for all three mutation sites (M815I, T917I and L920F mutations) has provided a complete set of monitoring tools for the efficient genotyping of head lice resistant to pyrethroids due to *kdr*. Using the SISAR kits, we confirmed that pyrethroid-resistant head lice due to *kdr* are prevalent in many locations globally. There is evidence, however, that some populations at the time of collection still possessed a substantial level of susceptible alleles and should respond to resistance management.

Overall, the data from current investigation suggest that lice from countries that have access to pyrethroid-based pediculicides have higher levels of *kdr*, perhaps due to overuse and/or misuse of them, than lice from countries that do not use pyrethroid-containing pediculicides. Thus, geographical location and the availability of pyrethroid-based OTC pediculicidal products likely played important roles in the frequency of *kdr* but the degree to which infested people are willing to cope with being infested may also impact the status of *kdr*.

For instance, the only head louse population that did not fit this trend was collected from the Navajo reservation in Pinon, AZ where 50% of the lice were determined as a RRR

diplotype, resulting in an overall 65% *kdr* allele frequency in this population. Infested individuals from this location preferred to manually remove lice or simply coexist rather than treat pediculosis with OTC products. Given this behavior, it was expected that this population would possess lice largely susceptible to pyrethroid-based pediculicides. Our findings suggest, however, that this head louse population was repeatedly exposed to pyrethroid-based OTC products. A possible explanation for the unexpectedly high level of *kdr* alleles actually detected is that an infested individual from outside the reservation brought resistant lice to the reservation and they spread via human contact.

In Egypt, head lice were collected from a rural village and possessed six different diplotypes of which five (HSH, RSR, HSR, RHR and RSH) have not been reported previously (Fig. 5). SISAR analyses of the Egyptian population indicated that only five percent of the lice had a *kdr* allele at T917I mutation site, whereas 70% of the lice had *kdr* alleles at M815I and L920F mutation sites. This findings may imply that the lice, at least in this population, acquired both M815I and L920F mutations first, prior to acquiring the T917I mutation, possibly due to the fitness disadvantage associated with expressing the T917I mutation alone as suggested by Yoon *et al*.[18] To date, we have never identified a louse with the T917I mutation alone. Therefore, the T917I mutation is an important factor for screening *kdr* lice, since the T917I mutation has never been detected in the absence of the other two mutations (M815I and L920F). Further investigation, however, is necessary to test this hypothesis. Also, lice with only the M815I and L920F mutations need to be evaluated to determine if they are phenotypically resistant to pyrethroids without the T917I mutation.

Until recently, only lindane, an organochlorine, was used to treat head lice infestations in Thailand and South Korea.[25] Since there was little to no exposure to pyrethrin/pyrethroid-based pediculicides until recently, the selection of *kdr* genotypes in these populations, if introduced, has not occurred widely. Likewise, lice from Ecuador and Papua New Guinea had no *kdr* alleles, suggesting that either the *kdr* alleles have not yet been introduced to these populations or that the *kdr* alleles were not yet detectible since head louse infestations at the time of collection were not commonly treated with pyrethrin/pyrethroid-based pediculicides. However, additional assessments are necessary to determine current *kdr* status in these countries, as the number of sites sampled was limited.

Current treatment methods for pediculosis include topical application of pediculicide, wet combing, or oral therapy.[26] For head louse populations that were determined to have 100% *kdr* allele frequencies, the pyrethrin/pyrethroid-based pediculicides are likely no longer effective control agents and alternative strategies should be implemented. Possible alternatives include the OTC pediculicides containing malathion, an organophosphate insecticide, available in the U.K. and prescription products containing carbaryl, a carbamate.[27]

Head louse populations that had no *kdr* alleles have more options for treating pediculosis. For these geographical locations, however, it is crucial to closely monitor the status of *kdr* to prevent possible control failures with continued use of pyrethrin/pyrthroid-based pediculicides.

Due to the nondiscriminatory prevalence of head louse infestations globally and the widespread detection of pediculicide resistance documented over the past decade, a new, more efficient method for detecting the early stages of resistance is needed. As determined in this investigation, the predicted genotypes based on the SISAR ratios were consistent across all three SISAR kits for field-collected louse populations. Furthermore, SISAR can detect heterozygous individuals that play important roles in population dynamics at the early phase of resistance.

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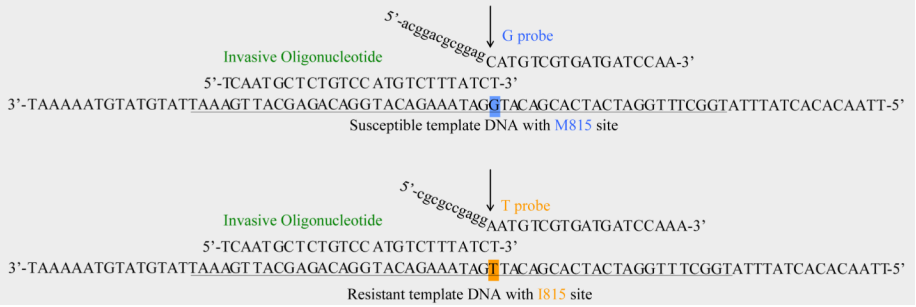

Figure 1.

Head louse VSSC gDNA sequence alignment with invasive oligonucleotide and specific primary G and T probes for the M815I mutation site. Underlined sequences indicate the 59 bp synthesized oligonucleotide targets specific for G and T probes, respectively. Arrows indicate site of cleavage.

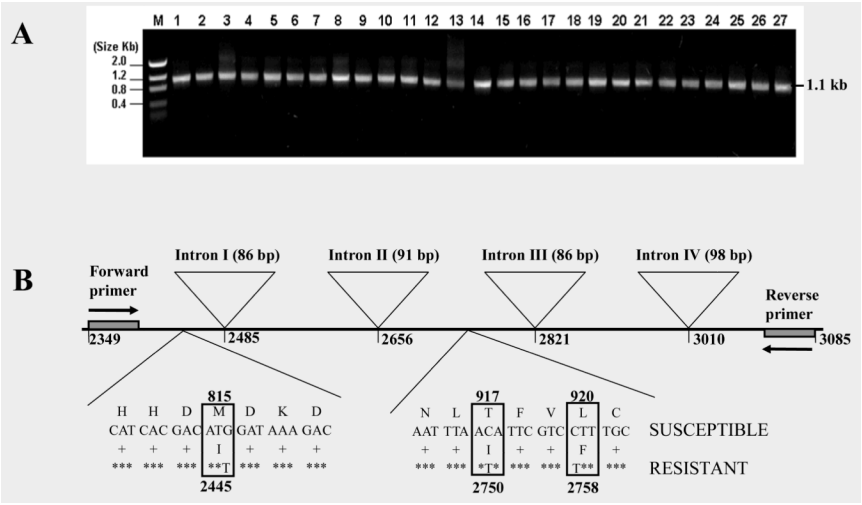

Figure 2.

1.1 kb-length PCR fragments amplified using TMS-HL gDNA extracted from 27 individual head lice (A). A diagram illustrating the 1.1 kb-length PCR fragment from head louse VSSC gDNA, including three point mutations (M815I, T917I, and L920F mutations), amplified using specific forward and reverse primers (B).

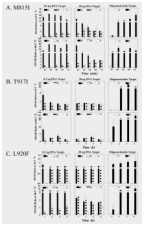

Figure 3.
Optimization of SISAR conditions using SISAR ratios at various DNA concentrations (0.2 and 20 ng/reaction), temperature (63.5 and 66.5 °C), and reaction time (15-75 min or 1-4 h). Control reactions using the oligonucleotide targets were performed similarly using the 1.1 kb PCR-amplified susceptible (with G or C nucleotide) and resistance (T nucleotide) DNA targets. (A) SISAR ratio for M815I. (B) SISAR ratio for T917I. (C) SISAR ratio for L920F.

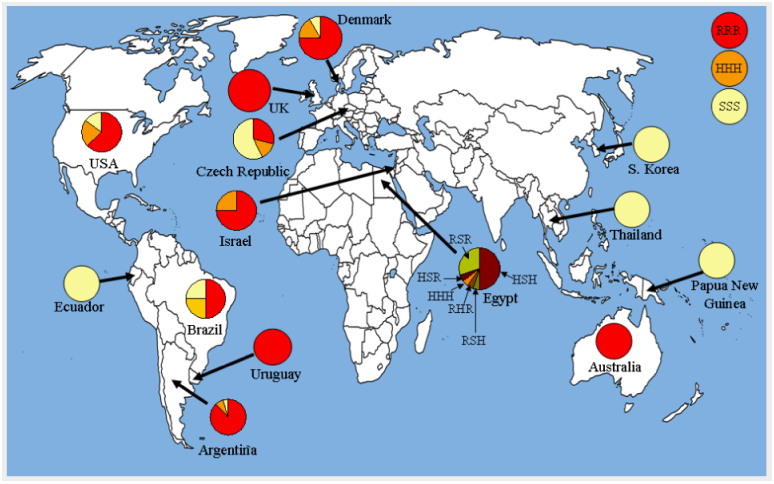

Figure 4.

Global *kdr* allele frequency map. Each pie chart shows diplotype proportions of lice collected from a country. Proportions of lice with resistance diplotype (RRR) are presented in red, proportions with susceptible diplotype (SSS) are represented in yellow, and proportions with heterozygous diplotype (HHH) are represented in orange. In Egyption population, six diplotypes are illustrated in different colors (HSH in brown, RSR in green, HSR in dark brown, HHH in orange, RHR in light brown, RSH in dark green).

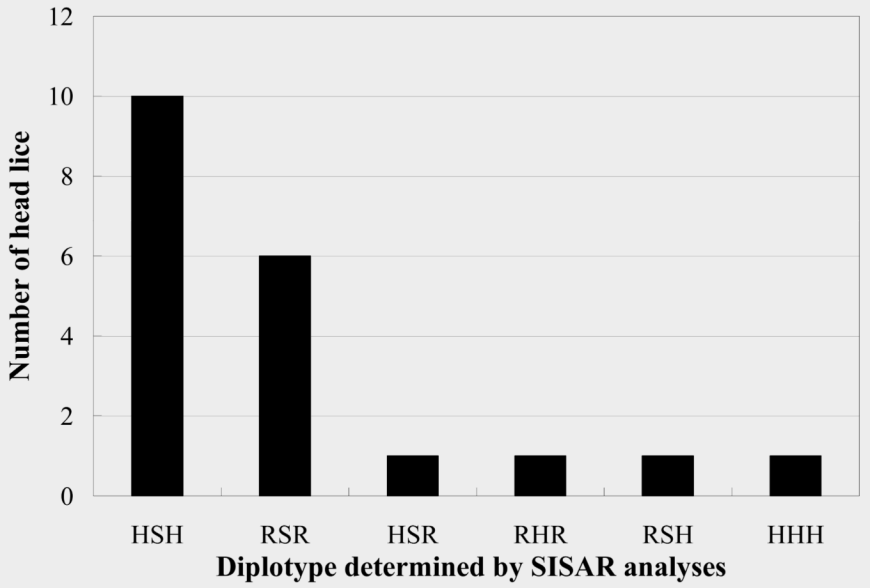

Figure 5.
Diplotypes of 20 Egyptian head lice collected from Kafr-el Sheikh Governorate determined by SISAR.

Table 1
Head lice collection for SISAR analysis

Geographical location of lice collection	No. of lice analyzed (No. of infested humans)[1]	Collectors, Collection month/Year
North America		
- U.S.A. (Pinon, AZ)	30 (12)	Jennifer Carroll[2], 05/2007
- U.S.A. (Ocklawaha, FL)	7 (1)	Katie Shepard[3], 12/2006
- U.S.A. (West Palm Beach, FL)	3 (3)	Katie Shepard[3], 12/2006
- U.S.A. (Saginaw, MI)	29 (2)	Jayne Heringhausen[4], 07/2007
- U.S.A. (Tracy, MN)	2 (1)	Mary Carter[5], 05/2007
- U.S.A. (Mathis, TX)	27 (1)	Leon Brown[6], 01/2001
- U.S.A. (San Antonio, TX)	9 (1)	Katie Shepard[3], 12/2006
South America		
- Argentina (Buenos Aires)	54 (ND) [7]	Maria Inés Picollo[8], 12/2006
- Argentina (Comodoro Rivadavia)	5 (ND)	Rita Kurdelas[9], 2006-2007
- Ecuador (Vilcabamba)	8 (23)	David Taplin[10], 02/2002
- Brazil	12 (15)	Jörg Heukelbach[11], 02/2008
- Uruguay (Montevideo)	8 (ND)	Carmen Rossini[12], 09/2007
Europe		
- Czech Republic	7 (ND)	Michael Kristensen[13], 2004
- Denmark (Uvelse)	12 (ND)	Michael Kristensen[13], 01/2004-03/2005
- U.K. (Bristol)	8 (3)	Gerald Coles[14], 06/2006
Africa		
- Egypt (Kafr-el Sheikh Governorate)	20 (ND)	Gamal Abo El-Ghar[15], 05/2008
Middle East		
- Israel (Jerusalem)	4 (12)	Kosta Mumcuoglu[16], 02/2008
Southeast Asia		
- South Korea (Seoul)	14 (ND)	Si Hyeock Lee[17], 02/2002
- South Korea (Hong-Sung)	20 (ND)	Si Hyeock Lee[17], 02/2002
-Thailand (Northwestern region)	33 (12)	Ratana Sithiprasasna[18], 04/2003
Oceania		
- Papua New Guinea	3 (1)	Dale H. Clayton[19], 7/2002
- Australia (Adelaide)	28 (ND)	Nicole Moore[20], 02/2005

[1] Number of infested humans who willingly provided the collected lice for SISAR analysis.

[2] Pinon Elementary School, Pinon, AZ, USA.

[3] Lice Solutions Network Inc., West Palm Beach, FL, USA.

[4]Saginaw County Department of Public Health, Saginaw, MI, USA.

[5]Tracy Area Public Schools, Tracy, MN, USA.

[6]Cacheaux, Cavazos, Newton, Martin & Cukjati, L.L.P., McAllen, TX, USA.

[7](ND), Not disclosed due to the human subject use protocols.

[8]Centro de Investigaciones de Plagas e Insecticidas, Buenos Aires, Argentina.

[9]The National University of the Patagonia San Juan Bosco, Argentina.

[10]University of Miami, Miami, FL, USA.

[11]Federal University of Ceará, Fortaleza, Brazil.

[12]University of the Republic of Uruguay, Montevideo, Uruguay.

[13]University of Aarhus, Kgs. Lyngby, Denmark.

[14]University of Bristol, Bristol, UK.

[15]Menoufiya University, Egypt.

[16]Hebrew University, Jerusalem, Israel.

[17]Seoul National University, Seoul, South Korea.

[18]US Army Medical Component of the Armed Forces Research Institute of the Medical Sciences.

[19]University of Utah, UT, USA.

[20]Environmental Health, City of Onkaparinga, Australia.

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Table 2

Validation of SISAR for genotype prediction using PCR products amplified from 27 TMS-HL head louse gDNA samples

	M815I mutation[1]			T917I mutation[2]			L920F mutation[3]		
Sample	**SISAR ratio[4] (SE)**	**PG[5]**	**AG[6]**	**SISAR ratio (SE)**	**PG**	**AG**	**SISAR ratio (SE)**	**PG**	**AG**
1	2.77 (0.49)	R	R	8.87 (0.34)	R	R	3.75 (0.20)	R	R
2	26.99 (1.74)	R	R	18.07 (1.97)	R	R	4.19 (0.29)	R	R
3	343.79 (6.43)	R	R	12.32 (0.78)	R	R	4.16 (0.03)	R	R
4	0.59 (0.03)	H	H	1.40 (0.04)	H	H	1.54 (0.17)	H	H
5	0.58 (0.03)	H	H	1.24 (0.05)	H	H	1.34 (0.18)	H	H
6	0.64 (0.03)	H	H	1.23 (0.01)	H	H	1.65 (0.14)	H	H
7	0.65 (0.01)	H	H	1.43 (< 0.01)	H	H	1.64 (0.05)	H	H
8	0.76 (0.10)	H	H	1.36 (0.01)	H	H	1.65 (0.07)	H	H
9	1.50 (0.60)	H	H	1.50 (0.02)	H	H	1.58 (0.13)	H	H
10	0.68 (< 0.01)	H	H	1.53 (0.01)	H	H	1.25 (0.18)	H	H
11	0.75 (0.05)	H	H	1.53 (0.03)	H	H	1.79 (0.14)	H	H
12	1.20 (0.04)	H	H	1.17 (0.03)	H	H	1.65 (0.06)	H	H
13	1.29 (0.09)	H	H	1.40 (0.04)	H	H	1.65 (0.06)	H	H
14	0.99 (0.23)	H	H	1.49 (0.01)	H	H	1.61 (0.08)	H	H
15	0.94 (0.26)	H	H	1.44 (0.06)	H	H	1.62 (0.04)	H	H
16	0.64 (< 0.01)	H	H	1.30 (0.07)	H	H	1.71 (0.06)	H	H
17	0.96 (0.24)	H	H	1.55 (0.11)	H	H	1.64 (0.05)	H	H
18	0.03 (0.01)	S	S	0.07 (0.01)	S	S	0.07 (< 0.01)	S	S
19	0.01 (< 0.01)	S	S	0.03 (< 0.01)	S	S	0.02 (< 0.01)	S	S
20	0.01 (< 0.01)	S	S	0.02 (< 0.01)	S	S	0.01 (0.01)	S	S
21	0.01 (< 0.01)	S	S	0.02 (< 0.01)	S	S	0.04 (< 0.01)	S	S
22	0.01 (< 0.01)	S	S	0.05 (< 0.01)	S	S	0.04 (< 0.01)	S	S
23	< 0.01 (< 0.01)	S	S	0.04 (< 0.01)	S	S	0.04 (< 0.01)	S	S
24	0.02 (< 0.01)	S	S	0.04 (< 0.01)	S	S	0.04 (0.01)	S	S
25	0.02 (< 0.01)	S	S	0.07 (0.01)	S	S	0.04 (< 0.01)	S	S
26	0.02 (< 0.01)	S	S	0.06 (< 0.01)	S	S	0.06 (< 0.01)	S	S
27	0.02 (< 0.01)	S	S	0.06 (< 0.01)	S	S	0.04 (< 0.01)	S	S

[1] SISAR-M815I was performed at 63.5 °C for 0.5 hr using 0.2 ng of template DNA per reaction.

[2] SISAR-T917I was performed at 66.5 °C for 1 hr using 0.2 ng of template DNA per reaction.

[3] SISAR-L920F was performed at 63.5 °C for 1 hr using 0.2 ng of template DNA per reaction.

[4] Criteria; S SISAR ratio ≤ 0.25, 0.5 < H SISAR ratio < 2.0, 2.5 ≤ R SISAR ratio.

[5] PG, predicted genotype by SISAR. R, resistant homozygote; H, heterozygote; S, susceptible homozygote.

[6] AG, acutual genotype determined by the automated DNA sequencing. R, resistant homozygote; H, heterozygote; S, susceptible homozygote.