Permethrin Resistance Due to Knockdown Gene Mutations is Prevalent in Human Head Louse Populations

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Abstract: Permethrin resistance in head lice is mainly conferred by the knockdown resistance (*kdr*) trait, a voltage-sensitive sodium channel (VSSC) insensitivity factor. Three VSSC mutations have been identified and confirmed to reduce the sensitivity of VSSC to permethrin. A step-wise resistance monitoring system has been established based on molecular resistance detection techniques. Quantitative sequencing (QS) predicts the *kdr* allele frequency in head lice on a population basis. The speed, simplicity and accuracy of QS made it an ideal candidate for a routine primary resistance monitoring tool to screen a large number of wild louse populations as an alternative to conventional bioassay. As a secondary monitoring method, real-time PASA (rtPASA) provides a more precise determination of low resistance allele frequencies. To obtain more detailed information on resistance allele zygosity, as well as allele frequency, serial invasive signal amplification reaction (SISAR) is utilized as an individual genotyping method. Using this approach, *kdr* alleles were detected in head lice from 10 of the 14 countries examined and an overall *kdr* allele frequency of 73.8% determined.

Keywords: Premethrin resistance, human head louse, Pediculus humanus humanus,resistance monitoring, knockdown resistance (kdr), resistance allele frequency.

INTRODUCTION

The pyrethrins and pyrethroids are the major commercially available pediculicides in the current market. Extensive use of the pyrethrins/pyrethroids as over-the-counter pediculicides, however, has rapidly lead to resistance. Pyrethroid resistance in head louse populations appears to be widespread in the United States and other countries but varies in intensity and is not yet uniform [1]. Thus, the establishment of proactive resistance management system is essential to maximize the life span of these major pediculicides prior to the complete fixation of head louse resistance. Loss of these valuable pediculicides in the current market due to the development of resistance would cause a serious problem in the control of pediculosis.

To elucidate the molecular mechanisms of permethrin resistance in the head louse, cDNA fragments that spanned the IIS4~IIS6 region of *para*-orthologous head louse voltage-sensitive sodium channel (VSSC) α-subunit gene were cloned and their sequences determined [2-4]. Sequence comparison between the permethrin-resistant and -susceptible strains identified three point mutations (M815I, T917I and L920F), all located in domain II, as putatively responsible for resistance (Fig. **1**). All three mutations were determined to exist *en bloc* as a resistant haplotype through sequence analyses of cloned cDNA and genomic DNA fragments from individual louse samples, both containing the three mutation sites.

Functional analysis of the mutations was conducted by using the house fly *para*-orthologous VSSC α-subunit as a surrogate channel. The three mutations were introduced into the house fly VSSC α-subunit cDNA individually or in combination, and each channel variant was heterologously expressed in *Xenopus* oocytes [6]. Two-electrode voltage clamp analysis of the sodium channel variants with different combinations of the mutations revealed that the M815I and L920F mutations reduced permethrin sensitivity 2-3 fold when expressed alone but the T917I mutation, either alone or in combination, virtually abolished permethrin sensitivity. Thus, the T917I mutation plays a major role in permethrin resistance *via* a kdr-type nerve insensitivity mechanism, and can be used as a molecular marker for resistance detection

Detection of the early phase of resistance is crucial to the long-term, efficient management that can delay and reverse the development of resistance. However, early resistance detection is very difficult using conventional bioassay-based monitoring methods, particularly when resistance is recessive. In addition, collecting large numbers of live specimens, particularly in case of lice, is often impractical and always difficult. To circumvent these limitations, various individual genotyping techniques for the detection of resistance allele frequencies using genomic DNA extracted from target insects have been employed as alternative resistance monitoring tool [7, 8]. For the efficient monitoring of head louse resistance in the field based on resistant genotype, we have developed a set of three molecular tools, including quantitative sequencing (QS), real-time PCR amplification of specific allele (rtPASA) and serial invasive signal amplification reaction (SISAR). In this chapter, we describe and compare the features of individual techniques and how to employ them in routine resistance monitoring of head lice that is practical and cost-efficient.

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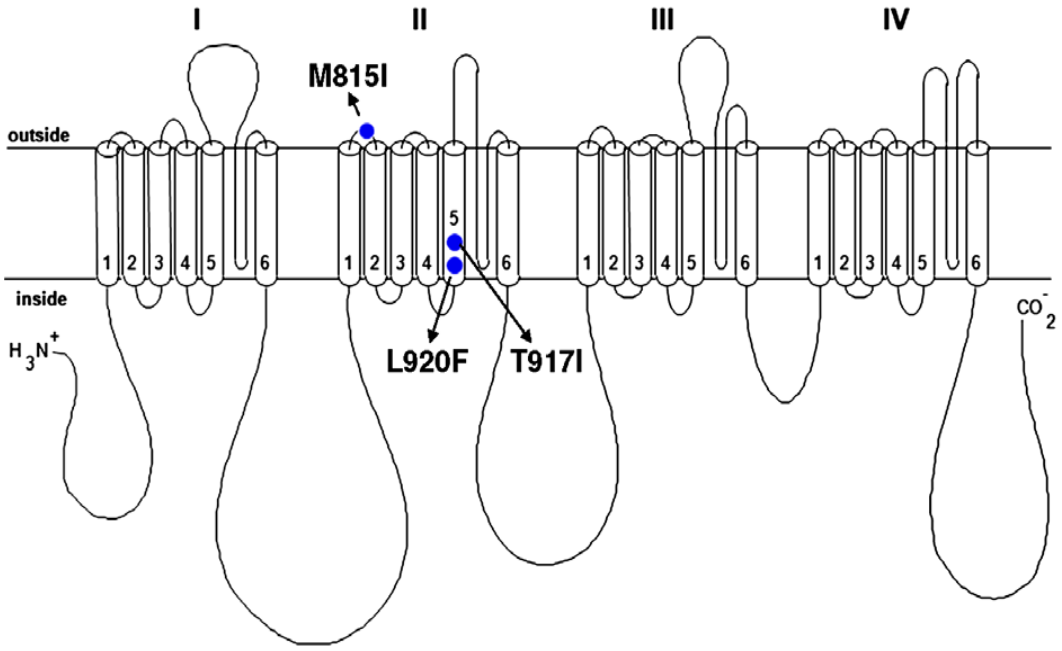

Fig. (1). Transmembrane topology of the voltage-sensitive sodium channel (VSSC) α-subunit showing the location of the three mutations responsible for knockdown resistance (kdr) in the human head louse. Reproduced with permission from ref. [5]. Copyright 2009 Elsevier Inc.

MATERIALS AND METHODOLOGY

Molecular Detection of kdr Mutations for Resistance Monitoring

Various individual genotyping techniques for the detection of resistance allele frequencies using genomic DNA extracted from target insects can be employed as resistance monitoring tools. For the effective monitoring of head louse resistance based on the *kdr* genotype, we have developed three molecular techniques; (1) quantitative sequencing (**QS**), (2) real-time PCR amplification of specific allele (**rtPASA**) and, (3) serial invasive signal amplification reaction (**SISAR**).

Quantitative Sequencing (QS). The QS protocol was developed as a population genotyping method for the prediction of *kdr* mutation frequencies (resistance allele frequency) in head louse populations [9]. Briefly, a 908-bp genomic DNA fragment of the VSSC α-subunit gene, encompassing the three mutation sites (M815I, T917I and L920F), was PCR-amplified from individual genomic DNAs (Fig. **2**). After verification of genotype, the PCR products with opposite genotypes were mixed together to generate standard DNA mixture templates with resistant allele frequencies of 0, 10, 30, 50, 70, 90 and 100% and sequenced by cycle sequencing. The nucleotide signal intensities of both resistant and susceptible alleles at each mutation site were determined from the sequence chromatogram (Fig. **3A**) and the signal ratios were calculated by dividing resistant nucleotide signal by the sum of the resistant and susceptible nucleotide signals. The signal ratios of template DNA mixtures were normalized by multiplying them with the normalization factor (signal ratio of the heterozygous DNA template/signal ratio of the 5:5 standard DNA template). A plot of the normalized signal ratios *vs* corresponding resistance allele frequencies were produced and standard regression equations were created for the estimation of resistance allele frequencies of unknown samples and their prediction intervals at the 95% confidence level (Fig. **3B**). Using the lower and upper 95% prediction equations, the average lower detection limits for the three mutations (M815I, T917I and L920F mutations) were determined as 7.4% at the 95% confidence level.

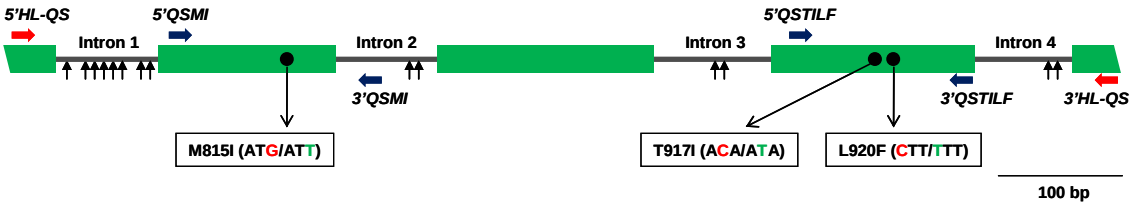

Fig. (2). Exon-intron structure of the 908-bp head louse VSSC genomic region that contains the M815I, T917I and L920F mutations. Shaded boxes and solid lines indicate exons and introns, respectively. Locations of three resistance mutations are marked with black circles. Vertical arrows indicate the approximate locations of intron polymorphisms. Horizontal arrows indicate the locations of the QS primers. Reproduced with permission from ref. [9]. Copyright 2009 Elsevier B.V.

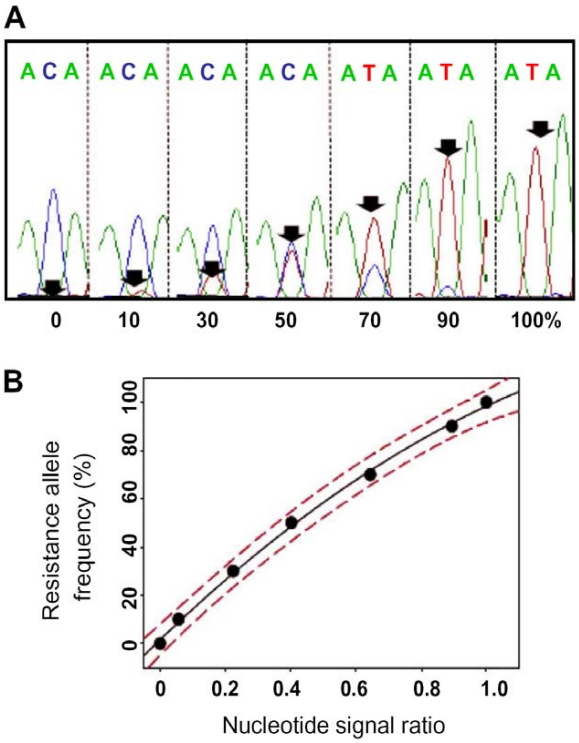

Fig. (3). Sequencing chromatograms of the standard template DNA mixtures with different resistance allele frequencies (**A**) and the plot of resistance sequence signal ratios *vs* resistance allele frequencies at the T917I mutation site (**B**). The intensities of the resistance allele nucleotide signals in the sequencing chromatograms are marked with an arrow in (**A**). The quadratic regression line is shown as a solid line with the upper and lower 95% prediction lines indicated by dashed lines in (**B**). Reproduced with permission from ref. [9]. Copyright 2008 Elsevier Inc.

Since QS is designed to use genomic DNA extracted and prepared from multiple louse specimens, it is suitable for processing a large number of louse populations. The use of a single DNA extraction from multiple louse specimens greatly reduces the overall cost and effort as repetitive DNA extraction from individual lice is arduous and costly. QS for 90 different population samples can be completed within 2 days in moderately equipped laboratories. The technique dependency of QS is also relatively low compared to other population genotyping techniques such as rtPASA-TaqMan [10] and rtPASA. Thus, the speed, simplicity and moderate sensitivity of QS make it an ideal candidate for a routine primary resistance monitoring technique to screen a large number of field-collected louse populations as an alternative to conventional bioassay. Since the sensitivity of QS is ca. 7.4%, a small to medium-size sampling (7-14 lice) per louse population may be sufficient and is practical, considering the difficulty of collecting a large number of louse samples. Taken together, prediction of resistance allele frequency by QS will greatly facilitate the initial resistance monitoring efforts in field-collected populations of lice.

Real-time PCR Amplification of Specific Allele (rtPASA). rtPASA is another protocol based on real-time PCR (rtPCR) for the prediction of resistance allele frequency on a population basis [11]. The rtPASA protocol utilizes the same genomic DNA template used for QS. If more precise determination of resistance allele frequency below the QS detection limit is required, rtPASA can be employed as a supporting monitoring step.

The standard DNA mixture templates for rtPASA were prepared as for QS except that different resistance allele frequencies were used (0, 1, 3, 8, and 16 %). Allele-specific primers matched the T917I and L920F mutation sites simultaneously (Fig. **4A**). rtPCR was conducted with resistant allele-specific primer set using Chromo 4^{TM} real-time detector (Bio-Rad, Hercules, CA), threshold cycle (Ct) values determined from each amplification curve, values normalized, and plotted against respective resistance allele frequencies (Fig. **4B**). Standard linear regression lines for the prediction of resistance allele frequency were generated by plotting the log scale of resistance allele frequencies *vs* Ct value (Fig. **4C**). Once the prediction equation is generated, resistance allele frequencies of unknown louse populations were estimated by incorporating Ct values into the equation.

rtPASA allows the detection of the *kdr* allele frequencies in head lice at the level as low as 1.13%. To detect the resistance allele frequencies lower than ca. 1%, however, a large-size sampling (50~100 lice per population) would be required. In addition, the technical dependency of rtPASA is relatively high compared to QS, requiring a well optimized protocol and experimental system to guarantee an accurate prediction.

Serial Invasive Signal Amplification Reaction (SISAR). Although both QS and rtPASA enable the prediction of resistance allele frequencies on a population basis, thereby allowing rapid screening of resistant populations, they do not provide information on allele zygosity (genotyping homozygous resistant RR, heterozygous RS and homozygous susceptible SS individuals). If information on resistance allele zygosity as well as allele frequency in a population is required, individual genotyping methods such as SISAR [12] can be conducted on a much reduced number of populations as a secondary or tertiary resistance monitoring step.

The SISAR (Fig. **5**) was originally developed for the high throughput analysis of single nucleotide polymorphisms using Cleavase®, a structure-specific endonuclease [13]. In the primary reaction, the invasive oligonucleotide anneals to both template DNAs. SNP-specific primary probes 1 or 2 anneal only to their complimentary template and the Cleavase enzyme cuts the 5' flap as indicated by the vertical arrows (Fig. **5A**). The cleaved 5' flaps bind only to their complementary FRET cassettes and results in the specific cleavage of the fluorophore from the respective FRET cassette (Fig. **5B**).

Information on allele zygosity is particularly useful for understanding the resistance population dynamics at the early stage of resistance where the resistance allele is present primarily as the heterozygous RS form in the population. SISAR requires, however, a large number of analyses (50~100 analyses of individual lice per population) to warrant accurate estimation of resistance allele frequency, which limits its applicability as a routine resistance monitoring technique.

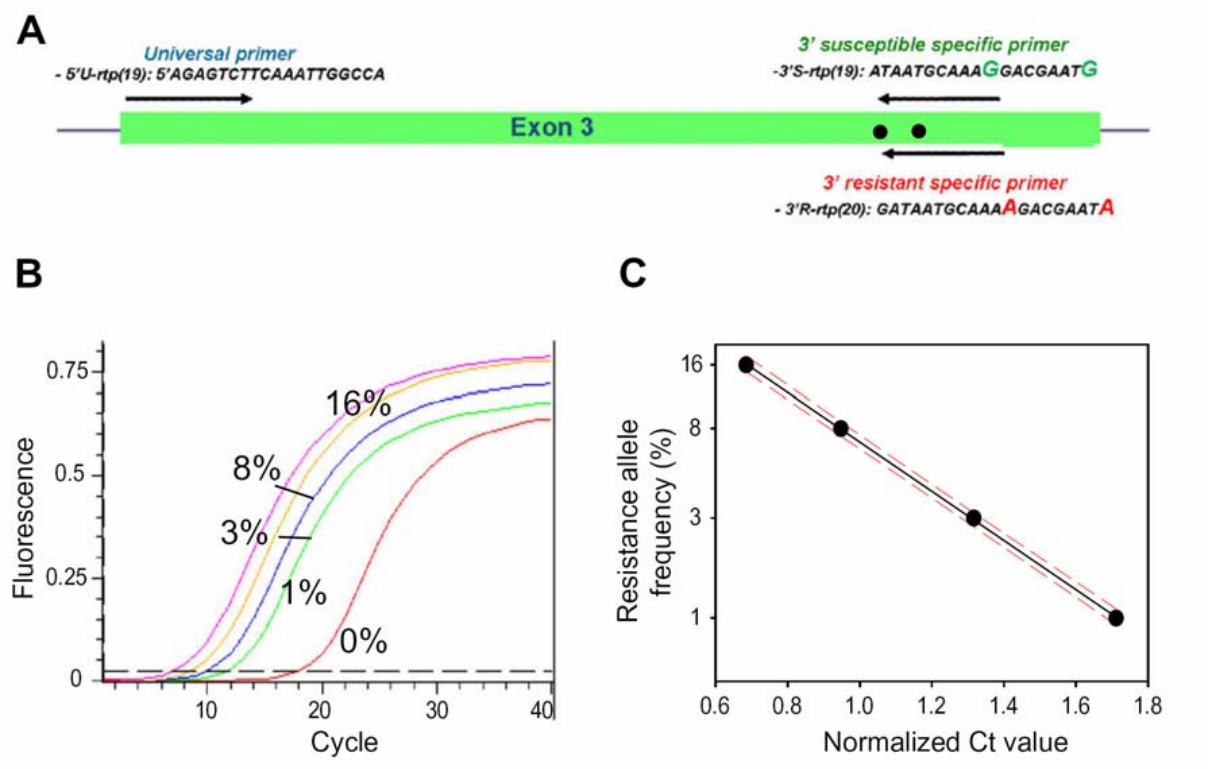

Fig. (4). rtPASA diagram (**A**), typical rtPCR amplification patterns using the DNA templates containing 0%, 1%, 3%, 8% and 16% resistant alleles (**B**) and the regression line generated from the plot of normalized Ct value *vs* the log of resistance allele frequency (**C**). Locations of the two mutations are marked with black circles and rtPASAprimers are indicated by horizontal arrows in (**A**). The regression line is indicated by a solid line with the upper and lower 95% prediction lines indicated by dotted lines in (**C**). Reproduced with permission from ref. [11]. Copyright 2009 ACS.

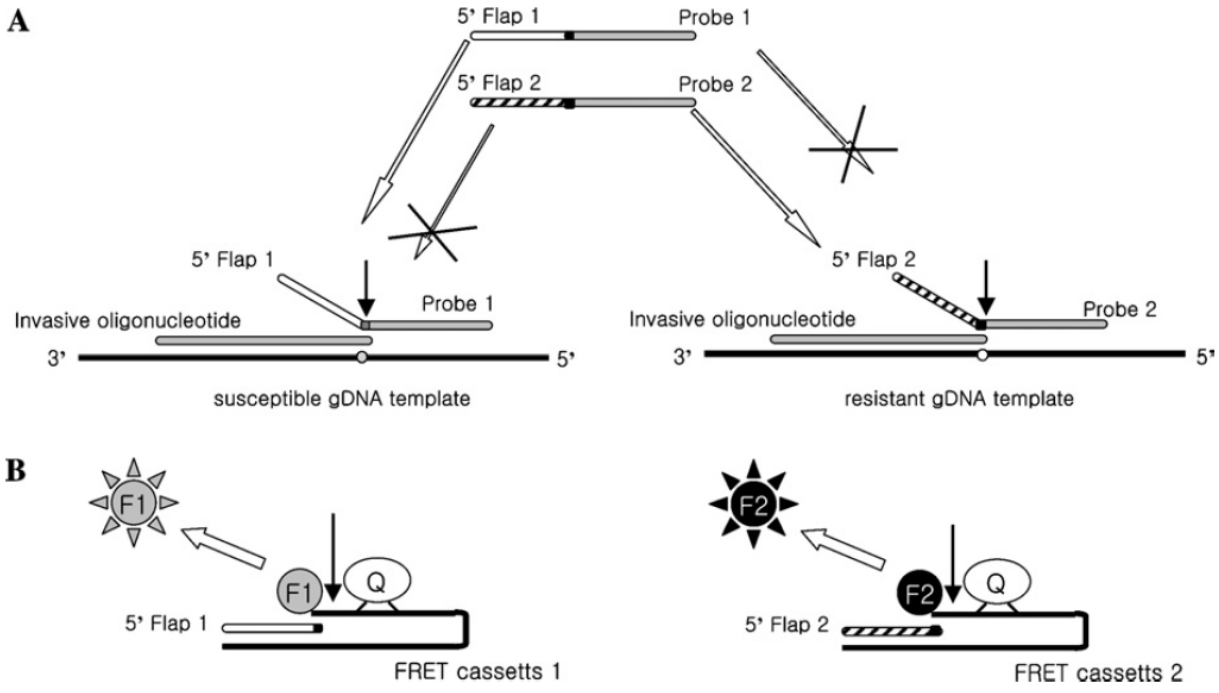

Fig. (5). Schematic of serial invasive signal amplification reaction (SISAR) based on matched enzyme (Cleavase)-substrate reactions. (**A**) is primary reaction. (**B**) is secondary reaction. Reproduced with permission from ref. [12]. Copyright 2004 Elsevier.

RESULTS

Determination of kdr Allele Frequencies and Allele Zygosity in Human Head Louse Populations Worldwide

Using these techniques, the resistance allele frequency and allelic zygosity of head louse populations collected from 14 countries worldwide were determined to construct a global *kdr* map (Fig. **6**).

Seven North American head louse populations were all collected from the U.S. The overall allelic zygosities within the U.S. populations were 64.4% RR, 21.35% RS and 14.25% SS with a calculated resistance allele frequency of 75.1%.

In South America, five head louse populations were collected. Head louse populations from Argentina and Brazil had allelic zygosities of 86% RR, 7% RS, 7% SS; and 50% RR, 25% RS, 25 % SS, respectively. The Ecuador population had 100% SS and the Uruguay population had 100% RR individuals. The calculated resistance allele frequencies were 89.5% for Argentina, 62.5% for Brazil, 0% for Ecuador, and 100% for Uruguay.

Three louse populations were collected within the European Union from the U.K., Denmark and the Czech Republic. Allelic zygosities were 100 % RR in the U.K., 75% RR, 17% RS, 8% SS in Denmark, and 22% RR, 11% RS, and 67% SS in the Czech Republic. Based on their allelic zygosity values, the U.K. population had a resistance allele frequency of 100%, 83.3% in Denmark, and 27.5% in the Czech Republic.

No resistant homozygotes (RR) were identified in lice from Kafr-Elsheikh Governorate, Egypt. These lice only possessed 15% RS and 85% SS individuals, resulting in a resistance allele frequency of 7.5%.

No susceptible homozygote (SS) lice were identified from Israel. The allelic zygosity values of these lice were 75% RR and 25% RS, resulting in a resistance allele frequency of 87.5%. All head lice collected from South Korea, Thailand, and Papua New Guinea were 100% SS. Comparatively, all head lice (n = 28) collected from Australia were 100% RR.

DISCUSSION

QS-based population genotyping can process a large number of louse populations simultaneously for the evaluation of resistance allele frequencies. Thus, the speed, simplicity and moderate sensitivity of QS make it an ideal candidate for a routine primary resistance monitoring technique to screen a large number of wild louse populations as an alternative to conventional bioassay. rtPASA can be employed as a secondary or supporting monitoring step. This method enabled the detection of the *kdr* allele frequency in the head lice at a level as low as 1.13%. If the information on resistance allele zygosity as well as allele frequency in a population is required, the individual genotyping methods, SISAR, can be conducted on a much reduced number of

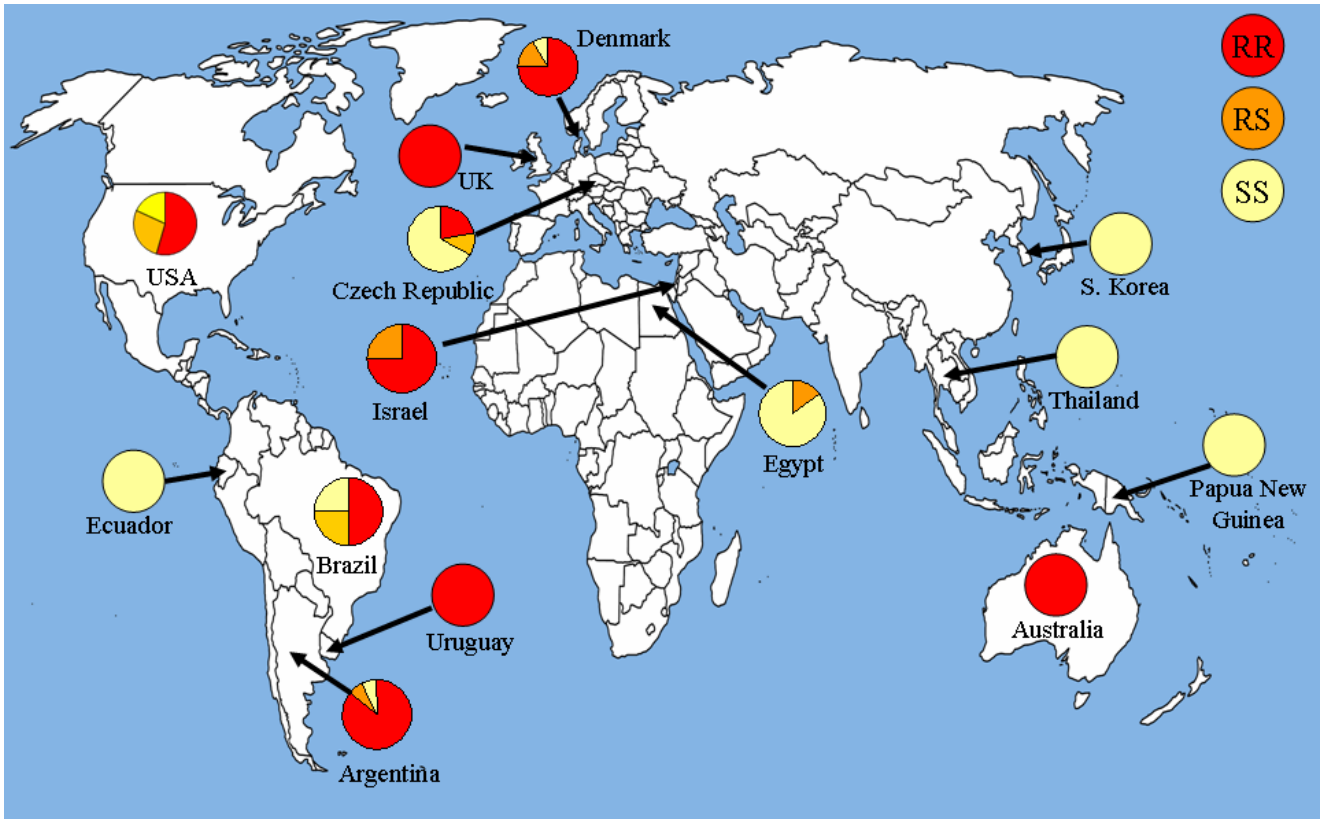

Fig. (6). Global occurance of permethrin resistant human head louse populations due to *kdr* mutations. Homozygous resistant populations (RR) are represented in red, homozygous susceptible populations (SS) are represented in yellow, and heterozygous populations (RS) are represented in orange. Reproduced with permission from ref. [14]. Copyright 2010 Wiley.

preselected populations as the secondary or tertiary resistance monitoring step.

In addition to detecting permethrin resistance mediated by the kdr trait, accumulation of yearly and regional databases on resistance allele frequencies will greatly facilitate the monitoring and understanding of resistance evolution patterns in different geographical regions over time. Based on the resistance allele frequencies estimated by these molecular techniques, differential actions for resistance management can be implemented. In regions where resistance allele frequency is saturated or near saturation, pyrethroids use should be curtailed and alternative pediculicides with different mode of actions used instead. In regions where the resistance allele frequencies are low or near zero, pyrethroids should be used cautiously and in conjunction with resistance monitoring program. This approach will extend the effective life span for this valuable group of pediculicides.

Recent completion of the human body louse (*P. h. corporis*) genome sequencing project allows us to acquire all the information on pediculicide target site genes and defense genes associated with pediculicide detoxification, and to use this information to effectively study resistance in the head louse. Interestingly, body lice have the smallest number of defense genes associated with metabolic resistance mechanisms (37 P450s, 12 GSTs, 18 Ests, 40 ABC transporters, etc.) among insects (Genbank http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=121224). The small number of defense genes facilitates the construction of a minimal and efficient microarray for the identification and transcriptional profiling of a more complete set of genes that are differentially expressed in pesticide-resistant strains and involved in pediculicide-induced tolerance. The identification of such resistance mechanisms and novel target sites may allow the development of resistance-breaking compounds (e.g. negative cross-resistance compounds) for improved louse control, more inclusive molecular diagnostics for effective and affordable monitoring in resistance management, and specific non-toxic synergists useful in novel strategies to control pediculicide-resistant populations [15]. In addition to the aforementioned molecular techniques to detect the known mutations responsible for reduced sensitivity of pediculicide target sites, complete understanding of detoxification mechanism will enable to establish molecular methods to detect metabolic resistance as well.

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