Geographical Distribution of Pyrethroid Resistance Allele Frequency in Head Lice (Phthiraptera: Pediculidae) From Argentina

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ABSTRACT The human head louse, *Pediculus humanus capitis* De Geer (Phthiraptera: Pediculidae), is an obligate ectoparasite that causes pediculosis capitis and has parasitized humans since the beginning of humankind. Head louse infestations are widespread throughout the world and have been increasing since the early 1990s partially because of ineffective pediculicides. In Argentina, the overuse of products containing pyrethroids has led to the development of resistant louse populations. Pyrethroid insecticides act on the nervous system affecting voltage-sensitive sodium channels. Three point mutations at the corresponding amino acid sequence positions M815I, T917I, and L920F in the voltage-gated sodium channel gene are responsible for contributing to knockdown resistance (*kdr*). The management of pyrethroid resistance requires either early detection or the characterization of the mechanisms involved in head louse populations. In the current study, we estimated the distribution of *kdr* alleles in 154 head lice from six geographical regions of Argentina. Pyrethroid resistance *kdr* alleles were found in high frequencies ranging from 67 to 100%. Of these, 131 (85.1%) were homozygous resistant, 13 (8.4%) were homozygous susceptible, and 10 (6.5%) were heterozygous. Exact tests for the Hardy-Weinberg equilibrium for each location showed that genotype frequencies differed significantly from expectation in four of the six sites studied. These results show that pyrethroid resistance is well established reaching an overall frequency of 88%, thus close to fixation. With 30 yr of pyrethroid-based pediculicides use in Argentina, *kdr* resistance has evolved rapidly among these head louse populations.

KEY WORDS head lice, knockdown resistance, sodium channel, *Pediculus humanus capitis*, insecticide resistance

Pediculosis capitis is the infestation of the human hair and scalp by the human head louse, *Pediculus humanus capitis* De Geer (Phthiraptera: Pediculidae). It causes scalp itching, irritability, and occasional secondary bacterial infection as a result of scratching (Roberts 2002). Moreover, this ancient medical problem generates emotional and social distress because of the associating stigma of lice with poor personal hygiene and poverty, school absentees because of "no-nit" policies, sleep disturbances, and difficulties in concentration, which can also lead to poor performance in school (Heukelbach and Feldmeier 2004). The potential of head lice as vectors of deadly pathogens is raising as a health concern because in recent years, head lice have been found to carry *Rickettsia prowazekii* and *Bartonella quintana*, the causative agents of typhus and trench fever, respectively (Robinson et al. 2003, Sasaki et al. 2006, Angelakis et al. 2011). In addition, *Acinetobacter baumannii*, a bacteria involved in hospital-acquired infections was detected in head lice from Paris (Bouvresse et al. 2011). The spread of lice occurs mainly through direct host-to-host contact (Roberts 2002). Head louse infestations are widespread throughout the world affecting mostly school-aged children (Falagas et al. 2008). It has been increasing since the early 1990s partially because of ineffective pediculicides (Burgess 2009). This inefficacy is because of a variety of reasons such as the sale of ineffective products, the incorrect use of pediculicides, and development of resistance to insecticides such as DDT, malathion, and permethrin in several countries (Burgess 2004, Mumcuoglu et al. 2009).

Pyrethroid insecticides are potent neurotoxicants affecting voltage-sensitive sodium channels (VSSCs). The VSSCs are integral membrane proteins responsible for the conduction of sodium ions that open or close the channels. When these channels function correctly, normal transmission of the nerve impulse occurs. On the contrary, when this process is altered by insecticides, intoxication symptoms (i.e., incoordination, tremors, paralysis, and death) are produced. Reduced neuronal sensitivity to pyrethroids and DDT,

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Table 1. Frequency of pyretroid resistance *kdr*-like alleles (T917I and L920F) in Argentinean head lice populations

Location	No. of lice analyzed (no. of infested subjects)	Genotype			Resistance allele frequency (%)	H-W[b] χ^2	F_{IS}[c]
		Observed S/S[a]	Observed R/S[a]	Observed R/R[a]			
Bahía Blanca	14 (2)	2 (14.3)	2 (14.3)	10 (71.4)	78.5	4.64*	0.60
Bariloche	17 (2)	0 (0)	1 (5.9)	16 (94.1)	97.1	0.02	—
Buenos Aires	68 (14)	6 (8.8)	5 (7.4)	57 (83.8)	87.5	29.97*	0.66
C. Rivadavia	15 (4)	4 (26.7)	2 (13.3)	9 (60)	66.7	7.35*	0.71
Cutral-Co	23 (2)	1 (4.4)	0 (0)	22 (95.6)	95.6	23*	1.0
Tucumán	17 (2)	0 (0)	0 (0)	17 (100)	100	—	—
Total	154 (23)	13 (8.4)	10 (6.5)	131 (85.1)	88.3	72.36*	0.66

[a] S and R are abbreviations for the susceptible and resistant alleles, respectively. Between brackets are the percentages of each genotype proportion within each population.

[b] Field populations were tested for the Hardy-Weinberg (H-W) equilibrium by the χ^2 ($P < 0.05$; df = 1; $\chi^2 = 3.84$).

[c] F_{IS} values >0 indicate heterozygote deficiency, while values <0 indicate heterozygote excess. A em dash (—) indicates values that were undefined.

*, indicates values that are statistically significant at $P < 0.05$. Significance level indicates rejection of the null hypothesis $F_{IS} = 0$ at $P < 0.05$.

termed knockdown resistance (*kdr*), is the result of the development of insecticide resistance populations and is one of the most common resistance mechanisms reported in insects (Hemingway and Ranson 2000). Selective mutations decrease the affinity of the insecticides in the putative pyrethroid-binding pocket on the voltage-gated sodium channel (Davies et al. 2008). The *kdr* trait is recessive, meaning that the genes that confer the trait are only expressed in homozygous individuals (Davies et al. 2008, Soderlund 2008).

Three point mutations at the corresponding amino acid sequence positions M827I, T929I, and L932F of the house fly *para*-orthologous VSSC (M815I, T917I, and L920F in the numbering of the head louse amino acid sequence) located in domain II conferring *kdr* are the most relevant factors in all DDT or pyrethroid-resistant head lice worldwide (Lee et al. 2003, Heukelbach 2010). These mutations are found together en bloc coexisting as a resistant haplotype (Lee et al. 2003, Kristensen 2005). The three louse mutations were inserted in all combinations using site-directed mutagenesis at the above mentioned corresponding amino acid sequence positions (i.e., M827I, T929I, and L932F) into the sodium channel gene of housefly; and these mutations were heterologously co-expressed in *Xenopus* oocytes with the sodium channel auxiliary subunit of housefly VSSC (Yoon et al. 2008). The authors found that each of the three point mutations causes a reduction in permethrin sensitivity. Moreover, when expressed alone, mutations M815I and L920F reduced permethrin sensitivity twofold to threefold, but the T917I mutation, both alone and in combination, virtually abolished permethrin sensitivity. Thus, the T917I mutation was identified as the primary cause of permethrin-resistance in head lice. The frequency of these mutations has previously been reported by several authors, with values that ranged from 10% in Japan (Kasai et al. 2009) to >90% in Denmark and France (Kristensen et al. 2006, Durand et al. 2011).

In Argentina, the overuse of products containing pyrethroids in the past three decades has led to the development of high levels of resistance in both eggs and motile stages (Picollo et al. 1998, 2000; Vassena et al. 2003; González-Audino et al. 2005; Mougabure-Cueto et al. 2008). However, all these studies were performed at the toxicological and biochemical levels of head louse populations from Buenos Aires. Thus, little is known of this resistance at the molecular level via *kdr*-type nerve insensitivity mechanism. The management of pyrethroid resistance requires either early detection or the characterization of the mechanisms involved in this resistance. The aim of this work was to investigate the presence and distribution of the T917I-L920F mutations in head lice from six geographic regions of Argentina.

Materials and Methods

Lice. Head lice were collected from heads of infested 6- to 12-year-old children, using a fine toothed antilouse comb. The protocol for louse collection was approved by the ad hoc committee of the Centro de Investigaciones en Plagas e Insecticidas (Research Center of Pests and Insecticides, Buenos Aires, Argentina), and archived in our laboratory. Lice were obtained from elementary schools distributed along different locations of Argentina (Table 1; Fig. 1). Live head lice were placed in 95% ethanol and stored at −20°C until DNA extraction was performed.

Genomic DNA Extraction. Genomic DNA was isolated from individual nymphal or adult head lice following Ascunce et al. (2013) protocol. In brief, each louse was cut in half using a scalpel, placed in a 1.5-ml centrifuge tube containing cell lysis solution and proteinase, and homogenized using sterile plastic pestle. DNA extraction was completed following manufacturer protocol with modifications of the wizard genomic DNA purification kit (PROMEGA, Madison, WI). Then, a dilution from the original concentration to ≈5–10 ng/μl was made.

PCR Amplification of Specific Allele. An assay with modifications based on Durand et al. (2007) to genotype each head louse for the presence of the T917I and L920F replacements in the sodium channel's S5 transmembrane segment of domain II was established. Polymerase chain reaction (PCR) was performed to amplify one fragment of a 332-bp of the voltage-sensitive sodium channel α-subunit gene spanning the codons 917 and 920. Reactions consisted of a

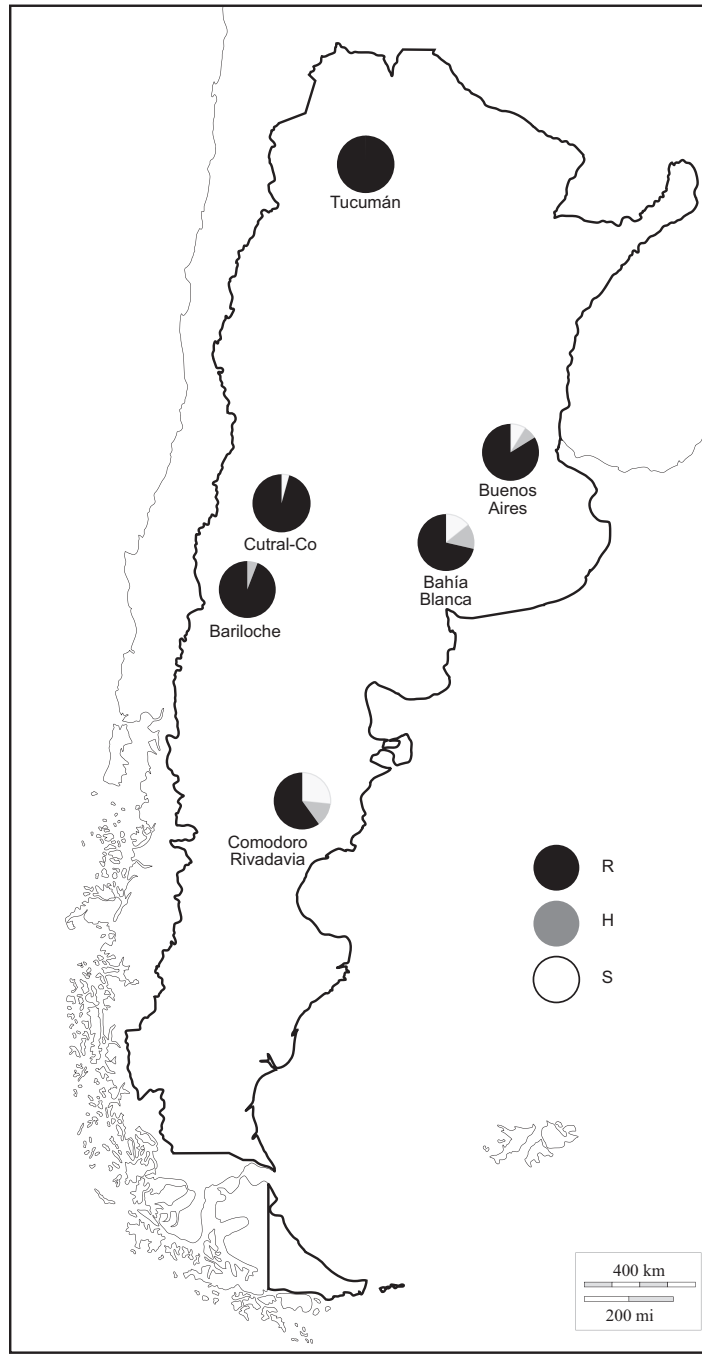

Fig. 1. Geographic distribution of the Argentinean human louse populations included in the current study. Each pie charts shows observed genotypes in different colors (RR in black, RS in gray, and SS in white).

total volume of 25 μl including 12.5 μl of MasterMix (PROMEGA, Madison, WI), 1 μl of each primer 5′-AAATCGTGGCCAACGTTAAA-3′ (sense) and 5′-TGAATCCATTCACCGCATAA-3′ (antisense), 2 μl of total genomic DNA, and 8.5 μl of water. The thermal cycling profile began with an initial denaturation at 94°C (10 min) followed by 40 cycles of 94°C (30 s), 56°C (30 s), and 65°C (1 min). Then, final primer extension was continued at 65°C (10 min). Amplified products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH). Sequencing was performed at INTA Castelar (Biotechnology department) using standard fluorescent cycle-sequencing PCR reactions (ABI3130XL, Applied Biosystems, Foster City, CA).

The sequences were assembled and analyzed with the Sequencher software V 4.1.4 (Gene Codes Corporation, Ann Arbor, MI). For each population, genotype frequencies were compared with Hardy–Weinberg expectations using the program Genepop (v. 4.2), Option one (Hardy–Weinberg exact tests), Suboption3 (probability test; Rousset 2008). Genepop was also used to estimate Wright's inbreeding coefficient (F_{IS}) using the method of Weir and Cockerham (1984), and for populations out of the Hardy-Weinberg equilibrium, these values were used to test for heterozygote deficiency and excess (Genepop Option 1, Sub-options 1 and 2, respectively) using the *U* test as described in Raymond and Rousset (1995).

Results

The frequency of the pyrethroid resistance gene was measured in 154 head lice distributed along several geographic regions of Argentina (Table 1; Fig. 1). All the six louse populations possessed *kdr*-like alleles with a high frequency ranging from 67 to 100%. Of these, 131 (85.1%) were homozygous resistant for pyrethroid target site sensitivity, 13 (8.4%) were homozygous susceptible, and 10 (6.5%) were heterozygous. On average, the frequency of the T917 and L920F mutations was 88.3%. The population of Comodoro Rivadavia had the highest percentage of susceptible individuals (26.7%), while Bariloche and Tucumán possessed no susceptible individuals.

If we considered that the *kdr* mutation is only partially recessive and functionally more significant in the homozygous state, the frequency of *kdr* T917I and L920F homozygotes ranged from 71.4 to 100%. Globally, the frequency of these mutations was 85.1%.

Exact tests for the Hardy-Weinberg equilibrium for each location showed that genotype frequencies differed significantly from expectation in four of the six studied places. Estimates of Wright's inbreeding coefficient (F_{IS}) indicated that genotype frequencies out of the Hardy-Weinberg equilibrium tended toward heterozygote deficiency (Table 1).

Discussion

This study examined *kdr* frequency distribution in *P. humanus capitis* of Argentina. The T917I and L920F mutations are present in all the geographically distant collecting localities with an observed mutant haplotype frequency of 0.86. Recently, Hodgdon et al. (2010) reported that some of the head lice collected in Egypt had the mutation T917I alone, in absence of both the mutations M815I and L920F. In the current study, all the analyzed head lice had both mutations coexisting en bloc as a haplotype as previously reported by several authors (Gao et al. 2003, Lee et al. 2003, Kristensen 2005, Thomas et al. 2006, Durand et al. 2007, Kasai et al. 2009). As mentioned by Kwon et al. (2008), *kdr*-like allele comprising the mutation T917I is a good and representative indicator of pyrethroid resistance in head louse populations.

Our results are consistent with the global head louse analysis performed by Hodgdon et al. (2010), in which the overall *kdr* allele frequencies in the United States, South America, European Union, Asia, Oceania, and Africa were of 74, 79.9, 75.9, 87.5, 100, and 47.5%; respectively. These findings support that pyrethroid resistance via a *kdr*-type nerve insensitivity mechanism is currently widespread but not uniform (Gao et al. 2003). Resistance alleles seem more prevalent in countries with an extensive use of pediculicides containing pyrethroids (Heukelbach 2010).

For the past 30 yr, pediculicides containing deltamethrin, permethrin, and d-phenotrin have largely been available in the Argentinean market against human head louse infestations. Despite several studies indicating that head lice from Buenos Aires showed high resistance levels (RR) >100 to permethrin (Picollo et al. 1998, 2000), pediculicides with pyrethroids as their main active ingredient are currently available in the market covering 30% of the total of the over the counter products.

Pediculosis is very common in elementary schools of Argentina. The overall prevalence of head louse infestation in several cities of Argentina varied from 29.7 to 61.4% (Gutiérrez et al. 2012), which is considerably higher than the 5% infestation value needed to be considered of epidemic importance (Clore 1988). Further, Delgado et al. (2010) found that 55% of the products used to treat head louse infestations in the Patagonian city of Comodoro Rivadavia contained pyrethroids. In the past 5 yr, the market share related to the sale of commercial pediculicides containing pyrethroids as their main active varied from 52 to 40% in Argentina. Thus, the selective pressure of pyrethroid-based pediculicides is still high in human head louse populations of Argentina.

Exact tests for the Hardy-Weinberg equilibrium showed that genotype frequencies differed significantly from expectation in four of the six studied populations. Thus, the deficiency of heterozygotes (10 per 154) and the elevated presence of homozygous *kdr* resistant mutations (131 per 154) in the studied populations suggest that the pyrethroid resistance is strongly established and almost in fixation. This is in accordance with what was found by other authors on head lice from France (Durand et al. 2011, Bouvresse et al. 2012). These authors reported that >93% of the head lice had homozygous *kdr*-type mutations, suggesting that the evolution of this phenomenon is not recent but strongly established within the studied populations. However, Thomas et al. (2006) found an excess of heterozygotes (77.2%) in head lice collected in schools from Wales, suggesting that the population is still under active selection pressure. It has been stated that *kdr*-type mutations in the VSSC possess little or no effect on the overall fitness of the individuals, which might result in a slow return of the resistant populations to the susceptible state (Roush and McKenzie 1987). It is remarkable that in a period of 7–8 yr, five permethrin-resistant head louse populations from Buenos Aires showed no variation in their toxicological response to permethrin (i.e., resistance

level values remained stable over time) (Toloza 2010). This is in accordance with several studies where pyrethroid resistance was maintained in horn and house fly field populations after the restriction or absence of pyrethroid use (Jamroz et al. 1998, Guglielmone et al. 2002, Huang et al. 2004).

Recently, Ascunce et al. (2013) analyzed the nuclear genetic variation at 15 microsatellite loci in human lice from North and Central America, Asia, and Europe. The authors reported a deficiency of heterozygotes relative to the Hardy-Weinberg equilibrium and high inbreeding values in most of the studied sites. Although they did not estimate the toxicological susceptibility to insecticides of these populations, they indicate that this genetic structure profile could be the result of an intensive selection by insecticides resulting in periodic population bottlenecks. These events might have reduced genetic polymorphisms in the insect genome following a parallel adaptive evolution model, in which some resistance alleles establish and evolve independently within populations. The human lice populations from Argentina seem to follow a similar genetic pattern of deficiency of heterozygotes relative to the Hardy-Weinberg equilibrium and high inbreeding values based on *kdr* alleles. However, it is still unknown whether pyrethroid resistance evolved once and then spread through the country or if it arose independently several times as a consequence of local artificial selection and further analysis need to be done.

Standardized toxicological and biochemical bioassay methods for evaluating the status and development of insecticide resistance are valuable in the characterization of the phenotype of populations to insecticides. However, they would be complemented by the PCR assay because molecular diagnostics allow the identification of heterozygote individuals, the determination of genotypes, and the phenotype and genotype relationship. An adequate integrated pest management program should consider monitoring the susceptibility to insecticides of exposed populations to detect early levels of insecticide resistance and promote preventive measures to avoid its spread. Further research should focus on the understanding of neutral genetic variation and their relationship with selective markers. In particular, sequencing genes that confer resistance allows us to investigate whether resistance to a specific insecticide or group of insecticides has single or multiple evolutionary origins and to understand the complexity of the genetic architecture of head lice from Argentina.

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