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Molecular analysis of pyrethroid resistance in *Pediculus humanus capitis* from Israel

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ABSTRACT

Head lice infestations are common amongst school children in many countries. In Israel, over the last two decades, control strategies have focused on the use of pyrethroids. Control failure was reported in several areas of the country two years after introduction of permethrin to the market. In the present study lice were collected from children from nine primary schools in six neighborhoods in Jerusalem. The overall prevalence of head lice infestation was 11%, while 93% of the examined lice were found to be resistant to permethrin. The 3 earlier described point mutations involved in a knockdown resistance, i.e., M815I, T929I and I932F were used to detect resistance. Lice with 3 mutated sites were the most dominant haplotype detected (60%), while lice with 2 mutated sites (T929I and I932F) accounted for 31% of those sequenced. All point mutations existed on both alleles of the voltage gated sodium channel α-subunit locus.

KEYWORDS: *Pediculus humanus capitis*, head louse, permethrin, resistance, Israel

INTRODUCTION

Head louse (*Pediculus humanus capitis* (De Geer)) infestations are prevalent worldwide and are especially common amongst school children [1]. Control using DDT led to the occurrence of widespread resistance. This treatment failure and concerns about insecticide toxicity resulted in the withdrawal of DDT as a head louse treatment from all developed countries. Pyrethroids are viewed as a relatively safe alternative, but worldwide evidence of resistance to this class of insecticide is emerging in head lice. Resistance to permethrin has been documented recently in several countries including Czech Republic [2], Argentina [3], United Kingdom [4, 5], France [6] and the United States [7].

In Israel, the pyrethroid permethrin was introduced in 1991 for the control of head lice and subsequently accounted for ~80% of pediculicides used between 1991 and 1995. First reports of permethrin control failure occurred in early 1993. Permethrin resistance was confirmed by bioassays on head lice collected school children in mid-1994 [8]. The two major routes by which insects acquire resistance to pyrethroids are by an increase in the rate of insecticide detoxification (primarily increased cytochrome P450 activity) and target site (nerve) insensitivity. Metabolic resistance was not detected in Israeli head lice suggesting that pyrethroid resistance was primarily due to nerve insensitivity [9]. Recently 3 point mutations characterized as knockdown resistance (*kdr*) in the voltage gated sodium channel α-subunit, were identified [10]. These mutations are located in the second trans

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membrane domain (M815I, T929I and I932F) and it was shown that when 2 of the sites (T929I and I932F) were mutated, resistance towards permethrin was demonstrated. Furthermore, all three has been suggested to exist as a haplotype [11].

The aim of this study was to study the prevalence of head lice in schoolchildren in Israel and to investigate the presence of permethrin resistance with the help of M815I, T929I and I932F mutations in lice. This research also aimed to develop a fast identification protocol for the 3 mutations.

MATERIALS AND METHODS

Study area

In the frame of a clinical study, nine primary schools from six different neighborhoods of Jerusalem, with a total number of 1,749 children, 6-14 years old, were examined for head louse infestation during the period December 2007 to March 2009. Permissions were obtained from the Helsinki Committee of the Hadassah Hospital in Jerusalem, the Ministry of Health and the Ministry of Education.

Collection of lice

The hair of each child was briefly combed with a regular comb to straighten the hair and then the entire scalp was combed with a louse comb (Innomed, Innomed Inc. Greenwich, CT) for a period of 3-5 min. In cases where the child's hair did not permit the use of a louse comb, the scalp was examined by hand for a period of 5-7 min. Lice stuck between the teeth of the comb, were removed with the help of an insect forceps and introduced immediately to 95% ethyl alcohol and were kept at 5-6°C until used.

Genomic DNA isolation

Genomic DNA was extracted from individual lice using 10 µl DNA release (Nippon Genetics Europe GmbH) per louse according to manufacturer's instruction with an increase, from 15 minutes to 30 minutes, during first incubation at 65°C.

FAST PCR amplification of genomic DNA

A FAST PCR protocol was developed in order to amplify 2 separate genomic DNA fragments, A and B. The A amplicon contained the M815I mutation, meanwhile amplicon B contain both the T917I and L920F mutation sites. CLC Bio was used to identify primer sites and Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA) was used in final primer design. The forward primer of amplicon A and reverse primer of amplicon B were synthesized with an 18-nucleotide M13 forward universal primer alternatively an 18- nucleotide M13 reverse sequence at the 5′ end respectively. The theoretical amplicon (excluding the 18 nucleotide M13 tail) were 113 nucleotides, A, and 132 nucleotides, B.

Primers listed in Table 1 were ordered from Operon Biotechnologies (Germany).

From the genomic DNA isolation 0.1 µl was added to a well in a MicroAmp optical 96 well reaction plate (Applied Biosystems) containing 5 µl AmpliTaq Gold 360 PCR 2xMaster Mix (Applied Biosystems), 2 pmol forward primer, 2 pmol reverse primer and water to a final volume of 10 µl. The FAST PCR was performed on a GeneAmp 9800 Fast PCR System (Applied Biosystems) with an initial denaturation temperature of 95°C for 5 minutes, followed by 35 cycles of 1 second at 96°C, 3 seconds at 60°C and 8 seconds at 68°C. Final elongation was set at 72°C for 20 seconds.

Table 1. Oligonucleotide sequences used.

Name	Sequence (5' to 3')
M13AForward	GTAAAACGACGGCCAGTACTTGTATTCGACCCATTCGTC
AReverse	CCACTCTTCAATGCTC
BForward	GAGTCTTCAAATTGGCCAAATCGT
M13BReverse	GGAAACAGCTATGACCATGTCCCATAACGGCAAATATGAA

FAST sequencing

Different methods to purify PCR products and to purify sequencing reactions are given. All methods have equal quality but different cost and time are invested. PCR products were prior to sequencing reaction either diluted 25-50 times in water or purified with the aid of exonuclease I and shrimp alkaline phosphatase. The enzymatic reaction was carried out in a MicroAmp optical 96 well reaction plate (Applied Biosystems) for each PCR product by addition of 10U of exonuclease I and 1U of shrimp alkaline phosphatase (both from Fermentas) to 5 µl of PCR product. The plate was set in a GeneAmp 9800 Fast PCR System, programmed to run incubation at 37°C for 15 minutes and enzyme inactivation at 85°C for 15 minutes.

From the diluted or purified PCR product 2 µl was used as template in both the forward and reverse sequencing reactions carried out in a MicroAmp optical 96 well reaction plate (Applied Biosystems). The template was added to a mixture of 8 µl containing 1 µl BigDye Terminator v1.1/3.1 Sequencing Buffer, 5X (Applied Biosystems), 2 µl BigDye ready reaction mix (Applied Biosystems), purified water and 4 pmol of M13 forward primer (GTAAAACGACGGCCAGT) alternatively M13 reverse primer (GGAAACA GCTATGACCATG). The sequence reaction was performed on a GeneAmp 9800 Fast PCR System (Applied Biosystems) with maximum ramping mode. The initial denaturation of the sequencing reaction was set to 96°C for 60 seconds, then 25 cycles of 96°C 0 seconds, 50°C 0 seconds and 60°C 30 seconds. Reaction ends with 4°C on hold.

Purification of the sequencing product was carried out by either ethanol precipitation or by BigDyeXterminator (Applied Biosystems). As a positive control M13 forward and reverse primer were used with KS+ Bluescript plasmid (Stratagene) as template in the sequencing reaction.

Purification of the sequencing products

Ethanol precipitation: A mixture of 27 parts 99.5% EtOH and 1 part 7,5 M ammonium acetate was prior to use mixed and stored at -20°C. The cold mixture, 28 µl, was added to 10 µl of sample in a 96 well plate. The plate was centrifuged at 4°C in a swing out rotor at 4000 g for 30 minutes and then directly centrifuged upside down at 20 g for 1 minute on a paper towel. To each well, containing precipitated sequencing product, 100 µl 70% ethanol was added and the plate was centrifuged at 4°C, 4000 g for 5 minutes. Again the plate was centrifuged upside down at 20 g for 1 minute on a paper towel and let to dry at room temperature for 10 minutes. Prior to sequencing analysis each well with purified sequencing product was dissolved in 10 µl water.

BigDyeXterminator: Purification of the sequencing product by BigDyeXterminator (Applied Biosystems) was carried out as stated by manufacturer. The sequences were analyzed on a 3130 *xl* Genetic Analyzer (Applied Biosystems). DNA sequences were analyzed by manual visual inspection of chromatograms using Lasergene, Seqman software (DNASTAR, Madison, WI).

RESULTS

Infestation with lice

Out of 1,749 children examined 192 (11.0%) were infested with lice and eggs, while 208 (11.9%) with nits only.

Resistance to permethrin

In order to examine the mutations in the kdr region, from each location 6 lice were tested, except from one school (TM) where only 2 lice were examined. Except from two schools (A1 and NB-1) all lice from same location demonstrated the same nucleotide sequence (Table 2). Taking into consideration that 2 out of 3 mutations are enough to become resistant towards permethrin, lice from all but one school (AI) were resistant towards permethrin or 93% of the lice tested were found to be resistant (Table 2). The majority of lice (60%) tested had all 3 described sites point mutated. The mutations could also be seen in both alleles. The second largest group (31%) was lice demonstrating mutation in 2 out of 3 sites. These lice are resistant towards the permethrin even if not yet mutated in position M815I. It was also observed that the mutations are coupled, i.e., the mutations T917I and L920F were always in pairs. If only 1 site was mutated it was always the site M815I.

Table 2. Mutations found on lice collected from children from nine primary schools in Jerusalem. From each sample 6 lice were tested separately. Within brackets the number of individual lice sequenced with a G or T.

School	Date of collection	No. of children from whom lice were collected	Total no. of lice collected	M815I, Wild-type G	T917I, Wild-type C	L920F, Wild-type C
AI	04.01.09	3	9	G/T (5/1)	C	C
DN	06.12.07	14	48	T	T	T
EF-1	20.01.09	6	21	T	T	T
EF-2	21.01.09	4	13	T	T	T
EF-3	24.03.08	10	39	A	T	T
ER-1	12.02.08	4	22	G	T	T
ER-2	13.02.08	11	40	G	T	T
HR	11.01.09	7	22	T	T	T
GE	07.12.07	15	44	T	T	T
NB-1	12.01.08	12	50	G/T (2/4)	T	T
NB-2	13.01.08	5	24	G	T	T
NB-3	14.01.08	3	20	T	T	T
TA	02.03.09	6	20	T	T	T
TM-1*	03.12.08	2	2	G	T	T
TM-2	18.02.08	7	34	G	T	T

*Only 2 lice collected and tested.

DISCUSSION

DDT was the mainstay of louse control in Israel until the early 1980's [12] when DDT resistance developed in local head lice [13]. Significant changes in susceptibility to permethrin were observed in head lice collected in 1989 and 1994 [8]. Biochemical assays of lice collected in 1989 and 1994 showed that DDT resistant populations were more heterogeneous for GST activity than the susceptible laboratory strain of body louse *Pediculus humanushumanus* L. A correlation in lice with high GST activity and high rates of DDT dehydrochlorination has been found, indicating a GST-based DDT resistance mechanism in field samples of *P. humanus capitis* (Hemingway *et al.* 1999). The presence of a metabolic DDT resistance mechanism does not however preclude co-selection of target site (kdr) and metabolic mechanisms. The fact that no elevation of esterase activity was observed in these lice shows that esterase-based metabolic pyrethroid-resistance mechanisms are not involved. Metabolism of monooxygenases was slightly, but not significantly, higher in pyrethroid-susceptible than pyrethroid-resistant lice [9].

Permethrin resistant head lice have been documented in France [6], the Czech Republic [2] and the UK [4]. Lately, differential susceptibility to permethrin has been shown in US head lice, where there is widespread use, compared to a population in Borneo where pyrethroids have not been used [7]. The *para*-orthologous sodium channel α-subunit fragment, which contains mutations associated with pyrethroid resistance in many insect species, e.g., the German cockroach [14], housefly [15] and in thrips [16] was amplified using a variant of PCR.

In the present study, sequence analysis confirmed the presence of three functional point mutations

(M815I, T929I and L932F) previously found in permethrin resistant louse samples [17]. A FASTPCR-based diagnostic protocol was developed to monitor the frequency of pyrethroid resistance in human lice populations in Israel. The use of this protocol enables us to determine louse individual nucleotide sequences in less the 2 hours. The major advantage of louse individual nucleotide sequence is that mutations can be distinguished as haplotype or diplotype.

Hodgdon *et al*. [18] showed that all three described mutations could coexist. However, it has been suggested that one mutation is enough to be shown as resistant phenotype, T929I [17]. Our study demonstrates that 34% of lice sequenced have 2 coexisting mutations, T929I and L932F.

CONCLUSIONS

The examination of lice from a large number of infested individuals in a big city such as of Jerusalem suggest that permethrin resistance could be quite widespread in Israel and this not only because of the high mutation rate and high percentage of resistant lice (93%), but also that none of the resistant lice were found to be resistant at only one allele.

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