Is resistance lousing things up in The Netherlands?

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Infestation with head lice (*Pediculosis capitis*) is a widespread nuisance. The Dutch National Institute for Public Health and Environment recommends combing possibly in combination with pediculicides. The current study aims to determine the levels of permethrin resistance of head lice in The Netherlands using both molecular tests and bioassays to develop evidence-based guidelines for the control of head lice in The Netherlands. For the latter an in vitro lice rearing system is under development.

Keywords: Pediculosis capitis, head lice, permethrin, resistance

Even though pediculosis capitis or infestation with head lice (*Pediculus humanus capitis*) is not a serious threat for human health, it is still a severe problem in The Netherlands (RIVM 2010). Infestation with head lice is often associated with poor hygiene while this is an incorrect assumption (Dodd 2001, Roberts 2002). The social stigma leads to distress, discomfort and embarrassment of both the infested child and parents (Lebwohl *et al.* 2007, Leung *et al.* 2005).

Head lice are very host specific insects which are specialized to live only on the human scalp (Heukelbach 2010, Meinking & Taplin 1996). It is unlikely to find head lice away from the human host since their survival off the scalp is only up to 48 h (Frankowski *et al.* 2010). Itching and irritation occur as consequence of an allergic reaction to the saliva of the head lice, although this allergic reaction does not always occur (Frankowski *et al.* 2010). The life cycle starts with an egg stage, followed by three nymphal stages which have a similar appearance as the final adult stage (hemimetabolous insects) (Meinking 2004). Eggs, or nits, are firmly attached to hairs at approximately 4 mm from the scalp, under warm and moist conditions in which the eggs can hatch (Frankowski *et al.* 2010). After day two of maturity, an adult female is able to mate and lay five to ten eggs per day, with a maximum number of 100-150 eggs during her approximately 20-30 day life (Meinking & Taplin 1996, Mumcuoglu *et al.* 2009).

Head lice are a persistent problem showing a constant low prevalence among schoolchildren in The Netherlands (Metsaars *et al.* 2000). Registered products for head lice control in The Netherlands contain either malathion (Prioderm®), permethrin (Loxazol®) or 4% dimeticone (XT Luis®) (RIVM 2010). In addition, the use of a louse comb for combing the hair of the infested child is strongly advised. Malathion belongs to the organophosphorous insecticides and kills head lice by inhibiting acetylcholinesterase (Gao *et al.* 2006, Meinking *et al.* 2007). Permethrin is a synthetic pyrethroid insecticide that affects the voltage-gated sodium channels of head lice resulting in disruption of the nervous system and inability to blood feed (Lebwohl *et al.* 2007). Unfortunately, resistance has developed against both malathion and permethrin resulting in strongly reduced effectiveness (Burgess 2009b, Meinking 2004). On the other hand, it is unlikely that resistance will develop against dimeticone and combing with a lice comb since both have a physical mode of action (Heukelbach *et al.* 2008). The mode of action of dimeticone is coating of the head lice which results in immobilization and which prevents excretion of water (Burgess 2009a). Physiological stress due to high osmotic pressure will lead to rupture of organs and eventually death of the head lice. The louse or nit comb, a fine toothed comb, is another control tool against head lice which can to be used on dry or wet hair of an infested person (Hill *et al.* 2005, Plastow 2001). With the louse comb all lice can be removed from the hair when done accurately. However, combing can be very time consuming and nits are difficult to remove.

Resistance (or decreased sensitivity) to both malathion and/or permethrin has already been reported from several parts in Europe, including UK, Czech Republic, Denmark and France (Downs *et al.* 1999, Izri & Briere 1995, Kristensen *et al.* 2006, Rupes *et al.* 1995, Silverton 1972). Relatively little research regarding pediculicide resistance in The Netherlands has been done so far. Though, treatment failure which could be due to resistance, has already been reported (Metsaars *et al.* 2000). However, resistance can be easily confused with reinfestation or incorrect use of products and guidelines (Frankowski *et al.* 2010, Heukelbach 2010). Several factors contribute to the development of resistance including, selection pressure of pediculicides, residual effects and incorrect use of pediculicides. (Insecticide Resistance Action Committee 2011, Lebwohl *et al.* 2007, Meinking 2004, Meinking & Taplin 1996). It is a misconception that resistant head lice cannot be killed by the pediculicide for which they are resistant; a higher dose or longer exposure time is required in order to kill resistant head lice compared to susceptible head lice (Bialek *et al.* 2011, Burow *et al.* 2010). Resistance should therefore be considered as relative lower sensitivity rather than absolute insensitivity against a certain pediculicide.

Three mutations (Table 1) have been identified in the voltage-gated sodium channel α-subunit gene of permethrin resistant head lice (Lee *et al.* 2000, 2003). These mutations in the so-called knockdown resistance (kdr) like gene result in

a certain level of nerve insensitivity and are therefore thought to be the main mechanism involved in permethrin resistance (Lee *et al.* 2003, Tomita *et al.* 2003). However, contradictory results have been found regarding the correlation between presence of kdr-like genes and clinical resistance against permethrin. Some studies indeed have found the correlation between the presence of mutations in the kdr-like gene and clinical resistance against permethrin (Bialek *et al.* 2009, Kasai *et al.* 2009, Kristensen *et al.* 2006, Yoon *et al.* 2003), while other studies found that permethrin is still an effective treatment against head lice with the resistance genes (Bialek *et al.* 2011, Burow *et al.* 2010). This indicates that presence of the mutations in the kdr-gene does not necessarily lead to absolute resistance to permethrin. More research regarding underlying mechanisms of permethrin resistance such as upregulation or overexpression of genes is necessary. However, the difference can also be due to differences in dose or exposure time, which are both important determinants of a successful treatment of resistant head lice.

Bioassays can be used to determine actual resistance levels against commonly used pediculicides. In common used bioassay head lice are exposed to the active compound of a pediculicide, such as permethrin, on filter papers (Burgess *et al.* 1995, Lee *et al.* 2000, Mumcuoglu *et al.* 1990, 1995, Picollo *et al.* 1998, Pollack *et al.* 1999, World Health Organization 1970). Results obtained with bioassays can be used to constitute dose-response curves in order to obtain insight in effectiveness of the tested component. However, one of the major difficulties associated with bioassays using head lice is the large number of lice which need to be exposed in order to obtain reliable results. Opposed to head lice, body lice (*Pediculus humanus humanus)* are often used for *in vitro* experiments; rearing body lice does often involve feeding on rabbits instead of human volunteers (Downs *et al.* 1999, Hemingway *et al.* 1999). However, in order to develop clear guidelines which are based on the Dutch situation, it is essential to use head lice originating from primary schools in The Netherlands. Two main methods that provide for a large number of head lice for bioassays are described in the literature. The first method involves the simultaneous large scale collection of head lice from schools after which the head lice are directly exposed to the to-be-tested products (Gao *et al.* 2003, Kristensen *et al.* 2006, Mumcuoglu *et al.* 1990, 1995, Picollo *et al.* 1998, 2000, Pollack *et al.* 1999), while the second method involves the development of an *in vitro* rearing system (Sonnberg *et al.* 2010, Takano-Lee *et al.* 2003, Yoon *et al.* 2006). Due to practical constrains such as time and number of volunteers, the first method was not applicable for this study and therefore attempts were made to set up an *in vitro* rearing system.

Before a head lice colony can be set up in the laboratory, head lice need to be reared on the arm of volunteers in order to obtain a large number of nits. This seems necessary since only a fraction (10.9-35.8%) of the newly hatched first instar nymphs are willing to successfully feed through the membrane in the sys-

tem, in contrast to later stages which are even more difficult to rear in the system (Takano-Lee *et al.* 2003). Due to the low feeding success there appears to be a strong selection of head lice which are able to feed in the system. This selection may lead to a laboratory population which is distinct from the wild-type head lice found on primary schools (Sonnberg *et al.* 2010). This could potentially lead to biased results obtained with the laboratory colony in bioassays. Although this selection may occur, such head lice colony is more suitable for research than body lice which are far more distinct from the population in The Netherlands.

The current study aims to determine the levels of permethrin resistance of head lice in The Netherlands using both molecular tests and bioassays in order to develop evidence-based guidelines for the control of head lice in The Netherlands. For realization of the latter, an *in vitro* head lice rearing system analogue to the system described by Yoon *et al.* (2006) has been developed. Due to large-scale development of resistance against permethrin, it is expected that Dutch head lice have become less sensitive for products containing permethrin (Braks *et al.* 2011, Lee *et al.* 2000, Metsaars *et al.* 2000, Mumcuoglu *et al.* 1995, Yoon *et al.* 2006).

MATERIALS AND METHODS

Specimen collection

In 2010, 1,000 primary schools in The Netherlands were solicited by email to enrol in this study. All schools were stimulated to inspect their pupils for head lice infestations at the first Wednesday after spring holidays. Furthermore, it was requested to send the collected head lice in small tubes to the Dutch National Institute for Public Health and the Environment (RIVM) in Bilthoven, The Netherlands. All received tubes were stored at −20 °C. All head lice collected during this campaign were used for molecular experiments regarding resistance genes.

In 2011, a total of 12 primary schools in Bilthoven and De Bilt, The Netherlands, were again solicited by email to enrol in the current study. The schools were requested to report infestations with head lice, after which the investigator directly visited the school. Infested pupils were combed with a louse comb (Nitcomb-M2®, Kernpharm, Veghel, The Netherlands) in order to collect living head lice and viable nits. All collected head lice and nits were put in a small cup with sealable lid, which was placed inside a small Styrofoam box, in which they were transferred to the laboratory situated at the RIVM. All living head lice and viable nits were used for setting up an *in vitro* rearing system

Rearing system

Adult head lice collected from schools were kept on the arm of the investigators (CV, MB, ET). A piece of extra thick blot paper (Biorad, Hercules, CA, USA),

mini blot size (7.0 × 8.4 cm), of approximately 2 mm thick was cut out at a size of 4 × 3 cm with an opening of 2 × 1 cm and was placed in the middle of an absorbent wound dressing (Leukomed®, BSN Medical, Hamburg, Germany) of 5 × 7.2 cm, from which the absorbent pad was removed. Head lice together with hair strands of 1-2 cm or a hair tuft were placed in the opening of the highly absorbent paper. The absorbent wound dressing was fixed to the arms of the investigators (Figure 1). Every one to three days, nits were removed and put into a small Petri dish with lid of 50 × 9 mm (Falcon® 1006, Becton Dickinson Labware, Lincoln Park, NJ, USA) containing a moist piece of filter paper, which was placed in an incubator (Heidolph unimax 1010 and inkubator 1000, Heidolph Elektro, Kelheim, Germany). Viable nits collected from schools were also placed into a small Petri dish with a moistened filter paper.

A rearing system analogue to the *in vitro* systems described by Takano-Lee *et al.* (2003) and Yoon *et al.* (2006) was set up in the laboratory of the RIVM. For this, the upper parts of 50 ml centrifuge tubes (Greiner Bio-One, Frickenhausen, Germany) were cut off at approximately 40-45 mm from the top (at the 35 ml mark). The screw top of the tubes served as blood reservoirs while the inverted tubes were used as rearing vessels (Figure 2). Tubes were washed with fabric softener diluted in water in order to reduce static electricity. A membrane was formed by putting 0.06 g of aquarium sealant (Aqua-sil, Den Braven Sealants, Oosterhout, The Netherlands) between two pieces of Parafilm M (Pechiney plastic packaging, Chicago, IL, USA) of 2.5 × 2.5 cm. The aquarium sealant was spread between the two pieces of Parafilm M by making rolling movements with an applicator stick. The membrane was stretched out to approximately 10 × 10 cm and fixed onto the screw-top end of the tube in order to seal the rearing

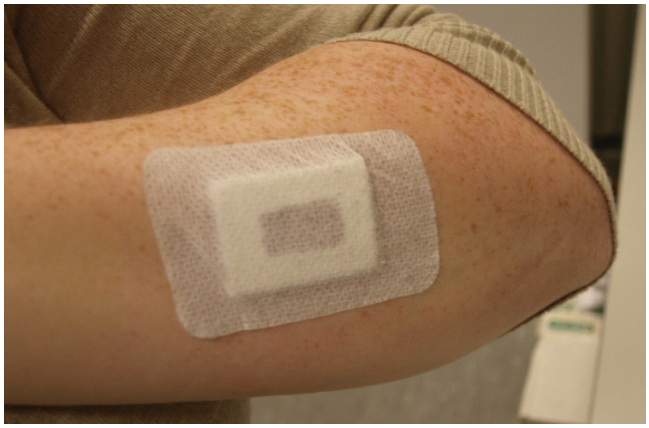

Figure 1. Feeding unit on the arm of the investigator.

Figure 2. All components of the *in vitro* rearing unit; rearing vessel, blood reservoir, hair tuft and piece of wide mesh gauze.

vessel. Hair tufts consisting of 200-300 human hairs with a length of 2.0-2.5 cm were made and pieces of wide mesh gauze with a diameter of approximately 2.6 cm were cut out (Figure 2). Every week, human blood provided by the researcher (CV) was added to blood tubes (3 ml) containing 9NC coagulation sodium citrate 3.2% (Vacuette sandwich tube, Greiner Bio-One, Kremsmünster, Austria). A mixture of 3,980 µl of the blood and 20 µl penicillin-streptomycin antibiotic mixture (1,000 parts penicillin, 1 mg streptomycin and 0.9 mg NaCl per ml) (Gibco®, Invitrogen, Carlsbad, CA, USA) together with a small magnetic stirrer were put into the blood feeding reservoir after which the rearing vessel was placed onto the lid. The rearing unit was sealed with Parafilm M in order to prevent microbial contamination of the blood mixture. The wide mesh gauze (Heltiq, Koninklijke Utermöhlen, Wolvega, The Netherlands) and hair tufts were placed inside the rearing vessel in order to mimic the human scalp. Nits, which were about to hatch, were placed inside the rearing unit. A piece of water-soaked filter paper (No. 1 grade 1 circles, Whatman Nederland, 's Hertogenbosch, The Netherlands) was attached to the upper part of the rearing unit with a paperclip after which it was sealed with Parafilm M, in order to maintain high humidity inside the rearing unit. The complete constituted rearing units (Figure 3) were placed onto a magnetic stirrer (Framo®-Gerätetechnik M20/1, Salm&Kipp, Breukelen, The Netherlands) in an incubator in order to maintain a constant temperature of 31 ± 1 °C. Rearing units were replaced every 48-72 h.

Detection of resistance mutations

A total number of 94 head lice originating from 19 different primary schools in The Netherlands were tested with conventional PCR for presence of the three mutations of the kdr-gene. DNA was extracted by boiling the individual head lice for 20 min at 95 °C in 100 µl ammoniumhydroxide (1 ml 25% ammonia + 19 ml H_2O) in closed Eppendorf tubes. The Eppendorf tubes were opened for an additional 20 min in order to evaporate the ammonia. The 908 bp long DNA fragment in the voltage-sensitive sodium channel α-subunit gene containing the three mutations (M815I, T917I and L920F) responsible for resistance against per-

Figure 3. Constituted rearing unit sealed with parafilm.

methrin was amplified with sequence specific primers (forward primer: 5'HL-QS 5'-ATTTTGCGTTTGGGACTGCTGTT-3' and reverse primer: 3'-HL-QS 5'CCATCTGGGAAGTTCTTTATCCA-3') (Kwon *et al.* 2008). Each PCR reaction consisted of 12.5 µl of HotStarTaq master mix (Qiagen Benelux, Venlo, The Netherlands), 2 µl of the template DNA, 0.2 µl of both primers (10 pmol/µl) and 10.1 µl of MiliQ water, in a total volume of 25 µl. The PCR program started with 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 63 °C and 1 min at 72 °C, ending with 10 min at 72 °C. PCR products were made visible with gel electrophoresis, on a 1.8% agarose gel. PCR products were purified using Exosap. Three different primer sets were used for sequencing the different mutation sites (T917I, L920F and M815I) and the entire 908 bp fragment (M815I site; forward primer: 5'-QSMI: 5'TGTGGCCTTACTTGTATTCGA-3' and reverse primer: 3'-QSMI 5'CCCCCCGCATTAAAATTAAAT-5', T917I and L920F sites; forward primer: 5'-QSTILF: 5'-AAATCGTGGCCAACGTTAAA-3' and reverse primer: 3'-QSTILF: 5'-TTACCCGTGTAATTTTTTCCA-5' and the whole 908 bp fragment; forward primer: 5'-HL-QS: 5'-ATTTTGCGTTTGGG ACTGCTGTT-3' and reverse primer: 3'HL-QS: 3'-CCATCTGGGAAGTTC TTTATCCA-3') (Kwon *et al.* 2008). Sequences of at least one of the three mutation sites were available for analysis of each head louse.

Statistical analysis

Life history traits of the reared head lice were analysed using Microsoft Office Excel 2003. Sequences of positive PCR products were blasted in GENBANK. Using Bionumerics version 6.6, all sequences have been analysed by manual visual inspection of the chromatograms in order to determine the genotype (susceptible or resistant) of the head lice.

RESULTS

Specimen collection

The maximum number of 140 primary schools enrolled in the 2010 campaign. Out of this maximum number, 31 schools (22%) returned a total number of 98 specimens (nits, nymphs and/or adults) to the RIVM (Braks *et al.* 2011). During later stages of this study, new samples were sporadically sent in. From only three schools viable specimen (nits, nymphs and or/adults) were collected during the 2011 campaign. From these schools a total number of approximately 30 nits (from which five nits hatched), 50 nymphs and 33 adult head lice were collected.

Rearing system

Head lice were successfully reared on the arm of the investigators. A total number of 1-10 head lice (first, second or third instar nymphs or adults) were placed inside one feeding unit on the arm of the investigator. Mating occurred fre-

quently, resulting in fertile nits, which were oviposited on the hair strands placed inside the unit, on fibers of the wound dressing or on the hair on the arm. The thick hair strands placed inside the units were preferred for oviposition. In this system, an average number of 4.5 ± 0.3 nits were laid by one female per day. Approximately 84.2% of the nits were viable, since development of an eyespot became visible. However, only 58.6% of the nits that showed development actually hatched in the *in vitro* rearing system, which is 49.3% of the total number of nits. It took 2-6 (average: 4.3±0.1) days before an eyespot, indicating the development of the nymph's nervous system, appeared. Up to now, nymphs have taken up blood through the membrane, although none of the nymphs inside the *in vitro* rearing system has reached the second nymphal stage.

Detection of resistance mutations

The genotype of a total number of 94 head lice originating from 19 different primary schools (Figure 4) in The Netherlands was determined in this study. Table 1 shows the nucleotides at the three mutation sites which determine the genotype (susceptible or resistant). Head lice showing a single peak at each mutation site are either homozygous susceptible or resistant, depending on presence of the mutation, while double peaks indicated heterozygosity (Gao *et al.* 2003, Kwon *et al.* 2008). All head lice included in the analysis appeared to be homozygous resistant for the three mutations in the kdr-gene. Figure 5 shows the three sections of the sequence containing the three mutations with single peaks, which are present in all homozygous resistant head lice tested in this study.

Table 1. Susceptible and resistant genotypes with the nucleotides at the three mutation sites.

Genotype	Mutation sites		
	M815I	T917I	L920F
Susceptible	ATG	ACA	CTT
Resistant	ATT	ATA	TTT

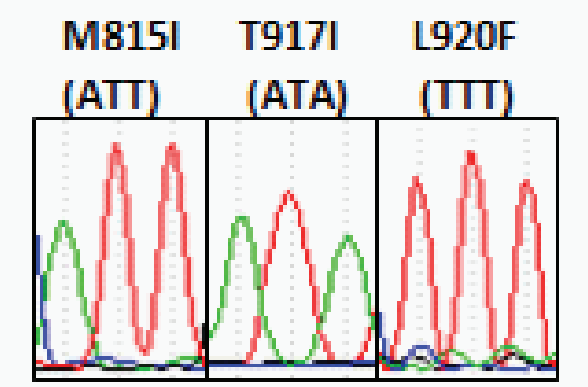

Figure 4. Origin of head lice with the homozygous resistant genotype.

Figure 5. Peaks at the three mutation sites of resistant head lice.

DISCUSSION

The main objective of this study was setting up an *in vitro* rearing system in order to enable future research on head lice resistance against recommended pediculicides, with the ultimate goal of developing clear national guidelines which can possibly lead to the eradication of head lice from The Netherlands.

During this study it appeared to be difficult to collect a reasonable number of living head lice from primary schools. First of all, response rates of schools reporting infestation with head lice were relatively low (only three out of 12 schools during 4 months). This could either be due to actual absence of head lice, lack of motivation to control pupils, lack of reporting minor infestations or because schools did not want to participate in this study. However, none of the schools indicated to refuse to participate in this study. Second, after checking the pupils of several classes at two schools which reported a recent outbreak of head lice, no living head lice or viable nits were found. An explanation for the absence of head lice in these classes could be the raised awareness of infestation with head lice being present among pupils and their parents in these classes. Proper treatment could have resulted in the eradication of head lice from these classes and therefore no head lice were found at the moment of arrival of the investigator. Interestingly, if infestation with head lice was ascertained, in most cases only one or two pupils per class were infested and only a few head lice were found on the child's head, which is in line with the study of Metsaars *et al.* (2000). Based on these findings, infestation with head lice appears to be a persistent problem which constantly maintains itself at low endemic levels among a low number of pupils at primary schools. Even though the number of infested children remains constantly low, parents and teachers experience the problem as serious. So far head lice have not been successfully eradicated from schools, making it a recurring problem which should be regarded as serious due to its

social aspects, despite the lack of severe health consequences.

During this study, some difficulties were encountered in developing an *in vitro* rearing system similar to the system described by Yoon *et al.* (2006). First of all, hatch rates of nits placed in the system were relatively low, although fertility was high. Approximately 84.2% of the nits appeared to be fertile since development was visible, however after development of the eyespot hatch rates were relatively low of 49.3% in contrast to hatch rates of 75% found by Takano-Lee *et al.* (2003). During the start of this study hatch rates were even lower (approximately 11.5%), which could be due to low genetic variation of the three parental head lice. The relatively low hatch rates could also be due to the humidity and oxygen supply inside the rearing units. In contrast to the units of Yoon *et al.* (2006), the rearing units in this study were sealed with Parafilm M in order to maintain high humidity, resulting in limited air supply. This could have had a suffocating effect on the nits. Second, only a limited number of head lice were collected during this study which resulted in relatively low genetic variation of parental head lice, since all head lice originated from one primary school. Third, static electricity inside the rearing tubes had a negative effect on nymph locomotion. This was overcome by washing the tubes with fabric softener. Fourth, Dutch head lice in general could be difficult to colonize as differences in colonization success between geographically distinct populations have been found by Takano-Lee *et al.* (2003). During this study period we did not succeed in setting up a self-maintaining laboratory colony of head lice. However, during this study newly hatched first instar nymphs successfully fed through the membrane of the rearing system, as blood has been observed in the abdomen of the nymphs, indicating that the system is functioning properly. Therefore, more time and ideally more adult head lice providing for nits are needed in order to set up a self-maintaining laboratory colony which is essential for performing bioassays. Alternatively, the effectiveness of pediculicide treatments could be evaluated in a clinical study as has been done by *e.g.* Burow *et al.* (2010).

All head lice tested for presence of the mutations in the kdr-gene appeared to have the homozygous resistant genotype. This is consistent with results obtained in other Western European countries including U.K. (100% homozygous resistant), Germany (93% homozygous resistant) and Denmark (75% homozygous resistant) (Clark 2010, Hodgdon *et al.* 2010). However, it remains unclear whether this resistant genotype actually leads to clinical resistance against permethrin. Due to practical constraints which needed to be overcome and lack of time, an insufficient number of head lice was available for testing with bioassays. Therefore, national guidelines for the control of head lice cannot be adapted and remain the same, based on available literature.

Conclusions

This study showed that 100% of head lice tested originating from The Netherlands has the homozygous resistant genotype. This indicates that pedi-

culicides containing permethrin (partly) have lost their efficacy in controlling head lice, but clinical proof is still lacking. More time is needed in order to rear a sufficient number of head lice which can be tested for permethrin resistance in bioassays. As clinical proof is lacking at this moment, national guidelines for the control of head lice cannot be adapted and should as yet continue to be based on literature. Another possible way of obtaining clinical proof is by focusing future research on clinical studies in order to determine efficiency of commonly used pediculicides.

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