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EXPANSION OF THE KNOCKDOWN RESISTANCE HUMAN HEAD LICE (PHTHIRAPTERA: PEDICULIDAE) FROM ORPHANAGES IN SOUTH SUMATERA (INDONESIA)

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Abstract

The head louse *Pediculosis capitis* affects all races and social levels, particularly those of low socioeconomic status. Currently, the mainstay treatment for pediculosis is insecticides such as permethrin but the extended use of permethrin worldwide has led to growing pediculicide resistance. There fore, this study investigated knockdown resistance (*kdr*) mutations in head lice populations from orphanages in three different localities in the Palembang (Indonesia). Forty-eight head louse samples were collected from twelve orphanages in the Palembang Subdistrict and subjected to PCR to amplify the α subunit of the voltage-sensitive sodium channel (*VSSC*) gene, *kdr* mutation (C ! T substitution). Restriction fragment length polymorphism (RFLP) patterns and sequencing were used to identify the *kdr* T917I mutation and demonstrated three genotypic forms including homozygous susceptible (SS), heterozygous genotype (RS), and homozygous resistant (RR). Of 48 samples, 32 (66,6%) were SS, 8 (16,7%) were RS, and 8 (16,7%) were RR and the overall frequency of the *kdr* T917I mutation was 0.31. The nucleotide and amino acid sequences of RS and RR also showed T917I and L920F point mutations. In conclusion, this is the first study detecting permethrin resistance among human head lice from orphanages in the Palembang Subdistrict of South Sumatra, Indonesia by PCR-RFLP. The study data may increase awareness of the increasing occurrence of the *kdr* mutation in head louse in orphanages in the Palembang Subdistrict within the province of South Sumatra, Indonesia.

INTRODUCTION

The head louse *Pediculosis capitis* is the most common ectoparasite globally and endemic to developed and developing countries in tropical and subtropical climates. Head lice are obligate blood-suckers which have the potential tocause anaemia. Itching caused by flea saliva can cause

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children to have difficulty sleeping and disrupted concentration, thereby decreasing educational achievement (3) (4). Typically, head lice infect more children from dysfunctional families, orphanages and special schools that live in dormitories. This infestation can cause severe pruritus on the patient's scalp resulting in sleep disturbance and scratching which could be followed by a secondary bacterial infection in the affected area. Head lice also impact daily life causing social embarrassment, loss of self-confidence, and school absence, particularly in school-aged children. In addition, *Rickettsia prowazekii*, *Bartonella quintana*, *Borrelia recurrentis*, and *Acinetobacter* spp. have been detected in the head lice but their role as a particular disease transmitter is still inconclusive (1, 2, 7,8). The prevalence of head lice infestation in Palembang varies from 15.1–88.4% depending on the sampling protocol used for head lice collection (5)(6)(9). School-aged females are particularly affected (10)(14) and the high prevalence of head lice infestation is partially correlated with lower socioeconomic status (14) and concentrated in rural compared to urban areas (5, 6, 9).

Permethrin, synthetic pyrethrin, is a commonly used over-the-counter chemical pediculicide and first-line treatment for *Pediculosis capitis*. It acts by binding to voltage-sensitive sodium channels in the nervous system of the head louse, causing muscle paralysis and death (2). Since permethrin has been widely used during the past decades, established evidence of permethrin resistance has been increasingly reported (13,14). Knockdown resistance (*kdr*) due to the three-point mutations (amino acid substitutions on the M815I, T917I, and L920F) has been identified in the α-subunit of the voltage-sensitive sodium channel (*VSSC*) gene(16, 18). M815I and L920F mutations reduce susceptibility to permethrin[17,19], whereas the T917I mutation alone or in combination with the L920F mutation plays an important role in permethrin resistance and can be used as a molecular biomarker for head lice permethrin or pyrethroid resistance (24, 27). Molecular analysis is one of the most popular techniques used to determine pyrethroid resistance in insects (20)(21), including quantitative multiplex sequencing (22,24), melting curve analysis genotyping coupled with quantitative PCR fluorescent resonance energy transfer technology (FRET) (25, 29), real-time PCR amplification of specific allele (rtPASA), serial invasive signal amplification reaction (SISAR) (2) , and restriction fragment length polymorphisms (RFLP) (17, 23, 32). Recurrent cases of head lice after treatment is still problematic in Palembang city, with a re-infestation rate after pediculicide treatment among school children in Indonesia of around 60% (31, 35). Evidence of permethrin resistance among head lice which could contribute to the increasing treatment failure rates in Palembang has not been investigated. Previous studies have demonstrated that PCR-RFLP is effective to demonstrate the genotyping of *kdr* T917I by using the *Ssp*I restriction enzyme (23,27,30), therefore, this study investigated the presence of *kdr* mutations in head louse populations collected from schoolchildren in orphanages in the Palembang Subdistrict within South Sumatra using PCR-RFLP and sequencing.

MATERIALS AND METHODS

The study was a descriptive survey design with molecular laboratory examination. A total of 48 head lice DNA samples were obtained from children in twelve orphanages in the Palembang province of South Sumatera (Indonesia). Forty-eight samples of tick DNA were collected from 48 Indonesia's orphanages.The DNA samples were stored at -80˚C at the Vector Biology and

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Vector-Borne Disease Research Unit, Department of Biomolekuler, Faculty of Medicine, Sriwijaya University and The Indolab Utama Jakarta.

Ethics statement

The study was approved and reviewed by the Health Research Ethics Committee of Health Polytechnic Ministry of Health of Palembang, No: 1244/KEPK/Adm 2/VIII/2021. The study was explained to each participant and written informed consent was provided by each parent or orphanage manager.

Head louse samples

A total of 48 head lice DNA samples were obtained from children in twelve orphanages in the Palembang province of South Sumatera, Indonesia. Forty-eight samples of tick DNA were collected from twelve subdistricts in Palembang city, namely Ilir Barat 1, Ilir Barat 2, Seberang Ulu 1, Seberang Ulu 2, Kemuning, Sako, Kalidoni, Ilir Barat1, Ilir Barat 2, Alangalang Lebar, Sematang Borang, Sukarame. The DNA samples were stored at -80˚C at the Vector Biology and Vector-Borne Disease Research Unit, Department of Biomolekuler, Faculty of Medicine, Sriwijaya University and The Indolab Utama Jakarta.

PCR amplification of the knockdown resistance *(kdr)* fragment

Conventional PCR was performed to amplify the α subunit of the *VSSC* gene, which contains the *kdr* mutation. Two oligonucleotide primers including kdr-F: 5′AAA-TCG-TGG-CCA-ACG-TTA-AA 3′ and kdr-R: 5′ TGA-ATC-CAT-T- CA-CCG-CAT-AA 3′ described by Durand et al. (26, 34,37) were used for the PCR amplification. The PCR reaction was performed in a final solution of 25 µl, consisting of 10X PCR buffer, 25 mM MgCl2 (Thermo Fisher Scientific, Waltman, MA, USA), 2.5 mM dNTPs (GeneAll Biotech, Seoul, Korea), 10 µM of each primer, 1 U *Taq* DNA polymerase (Thermo Fisher Scientific) and 3 µl of the DNA template using a PCR Mastercycler ProS (Eppendorf AG, Hamburg, Germany). The PCR cycling conditions consisted of an initial denaturation at 95˚C for 5 min, followed by 35 cycles of denaturation at 95˚C for 45 sec, annealing at 55˚C for 45 sec, extension at 72˚C for 1 min, and final extension at 72˚C for 7 min. Positive and negative controls were included and the expected PCR amplicons were approximately 332 bp in size. The amplified products were confirmed by electrophoresis in 1.5% agarose gel at 100 V for 30 min and then stained with ethidium bromide. The PCR amplicons were visualised with Quantity one Quantification Analysis Software Version 4.5.2 on a Gel Doc EQ system (Bio-Rad, Hercules, CA, USA).

Screening of the *kdr* mutation by PCR-RFLP

The PCR products were digested with the *Ssp*I enzyme to identify the *kdr* T917I mutation (substitution C ! T). The RFLP reaction mixture consisted of approximately 500 ng of PCR products, 10X buffer G, 10U *Ssp*I restriction enzyme (Thermo Fisher Scientific), and nuclease-free water to achieve a final volume of 10 µl. The reaction was initiated with incubation at 37˚C for 90 min, followed by heat inactivation at 65˚C for 20 min. The digested products were separated by 10% native polyacrylamide gel electrophoresis at 80 V for 90 min using the MiniProtein 3 cell (Bio-Rad, Hercules, CA, USA). Gels were stained with ethidium bromide and imaged on

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a Gel Doc EQ system (Bio-Rad, Hercules, CA, USA). To determine the *kdr* T917I mutation which is associated with pyrethroid resistance, RFLP was performed using the *Ssp*I restriction enzyme which recognises the AAT|ATT restriction site. When the T917I amino acid substitution occurs, the RFLP pattern should display two fragments in the RR genotype (261 bp and 71 bp) due to complete digestion and three fragments (332 bp, 261 bp, and 71 bp) in the RS genotype due to partial digestion and only one band of 332 bp in the SS genotype (undigested) (26,39).

Nucleotide sequencing

Direct DNA sequencing of the PCR products was performed to demonstrate the presence ofthe homozygous susceptible (SS), heterozygous (RS), and homozygous resistant (RR) genotype sequences. PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Direct sequencing was performed using the corresponding forward and reverse primers for the *VSSC* gene by a commercial sequencing company (Macrogen, Seoul, South Korea). To demonstrate the combination of both wild-type and mutant sequences in the RS genotype, the PCR products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) using a rapid DNA ligation kit (Promega) following the manufacturer's instructions. The ligated vectors were assembled for the transformation into competent cells (*Escherichia coli* DH5α), and then the recombinant plasmid DNA was screened using the blue-white colonies selection system. The white colonies suspected to contain the inserted gene were cultured and the plasmid DNA was extracted using the Invisorb Spin plasmid mini kit (STRATEC Molecular GmbH, Berlin, Germany). At least five clones of the RS genotypes were sent for sequencing by acommercial sequencing company (Macrogen, Seoul, South Korea) and the nucleotide sequences were analysed using BioEdit Sequence Alignment Editor Version 7.2.5 (37).

Statistical analysis of genotype frequencies

The frequency of resistance alleles was calculated by dividing the total number of resistance alleles by the total number of all alleles using a chi-square (28).

RESULTS

The *kdr* fragment was amplified in all head louse samples tested. The expected RFLP pattern of the homozygous susceptible or wild-type (SS) is one band of undigested PCR (332 bp), while the heterozygous genotype (RS) is expected to demonstrate three different fragments of 332 bp, 261 bp, and 71 bp. For the homozygous resistant or mutant (RR), the product is expected to show distinct fragments of 261 bp and 71 bp (Fig 1). Of 48 study samples, the SS, RS, and RR genotypes were found in 156 (60.00%), 58 (22.31%), and 46 (17.69%) samples respectively. Overall, the frequency of the *kdr* T917I mutation was 0.31 from the samples collected from twelve orphanages in the Palembang Subdistrict within South Sumatra, Indonesia. The frequency of the *VSSC* gene in the head louse population in this study ranged between 0.05 and 0.49 and the distribution of the*kdr* T917I genotype is shown in Fig 1.

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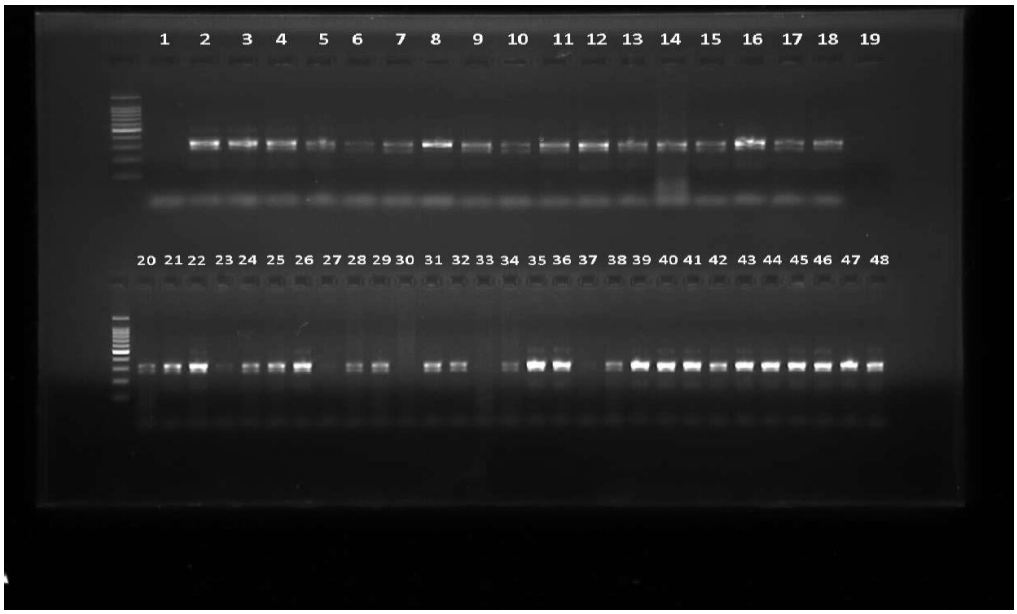

Fig 1: 10% native polyacrylamide gel electrophoresis demonstrates the RFLP patterns of *kdr* T917I genotypes

Lane 1 undigested PCR products, lanes 2, 6 and 9 are representative of the homozygous wild-type (SS). Lanes 3–5 of the heterozygous genotype (RS) have three bands. Lanes 7 and 8 are representative of the homozygous mutation (RR) with two bands. Lane M is a 50 bp DNA marker.

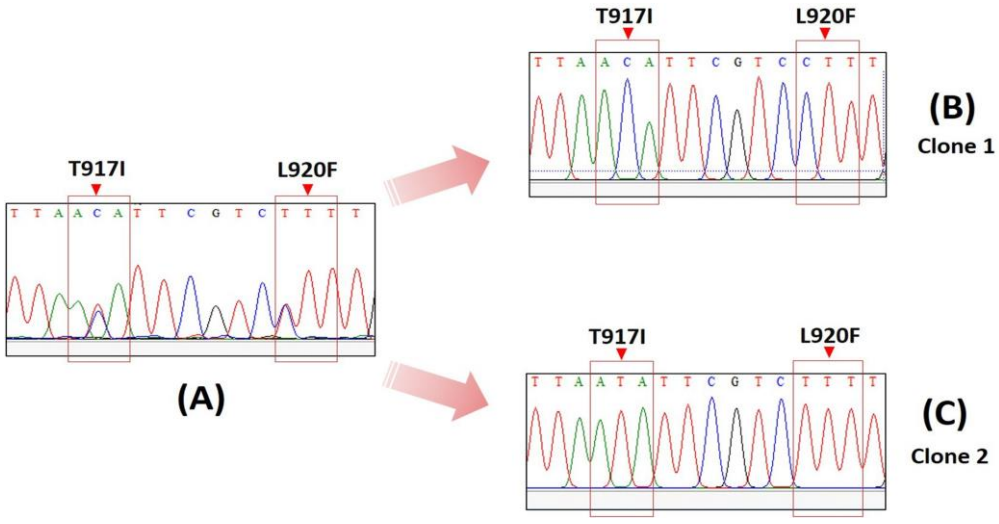

Fig 2: Chromatograms of heterozygous genotype (RS) from direct sequencing (A) equal signal of the nucleotide C and T at T917I and L920F are observed. Chromatograms of clones of the PCR products of the RS clearly demonstrate nucleotide C (B) or nucleotide T (C) signals, showing the *kdr* T917I and L920F of wild type and mutations, respectively.

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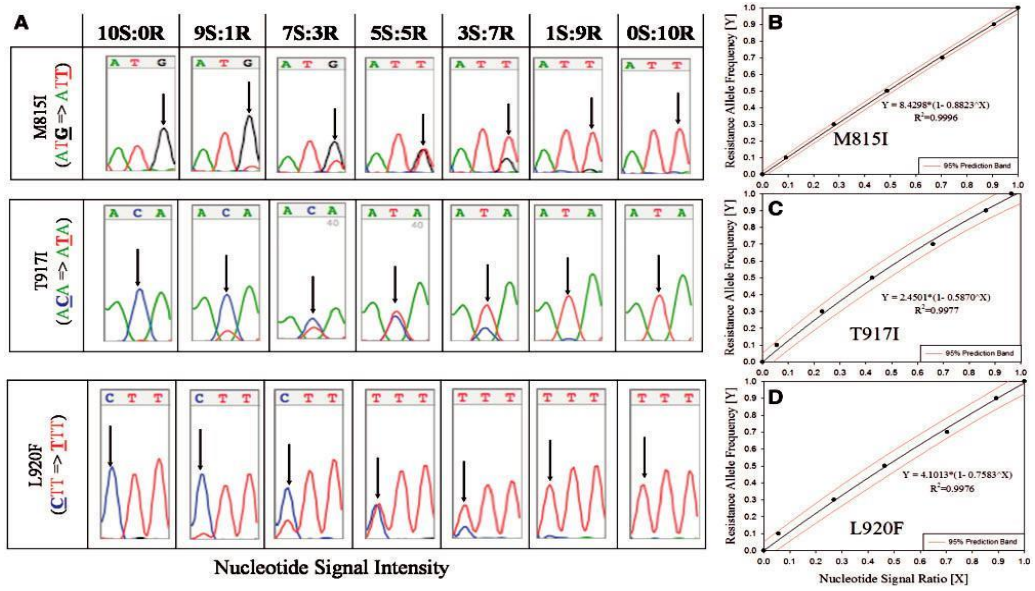

Fig 3: Generation of standard curves using quantitative sequencing for the determination of resistance allele frequencies (RAF) from collected lice. Nucleotide signal intensities for susceptible and resistant alleles at each mutation site (see arrows, Panel A, Fig. 2) were determined from the chromatograms, ratios calcu-lated, and plotted versus RAF to yield standard regression equations (Panels B–D, Fig. 1) and used to calculate RAF from collected samples

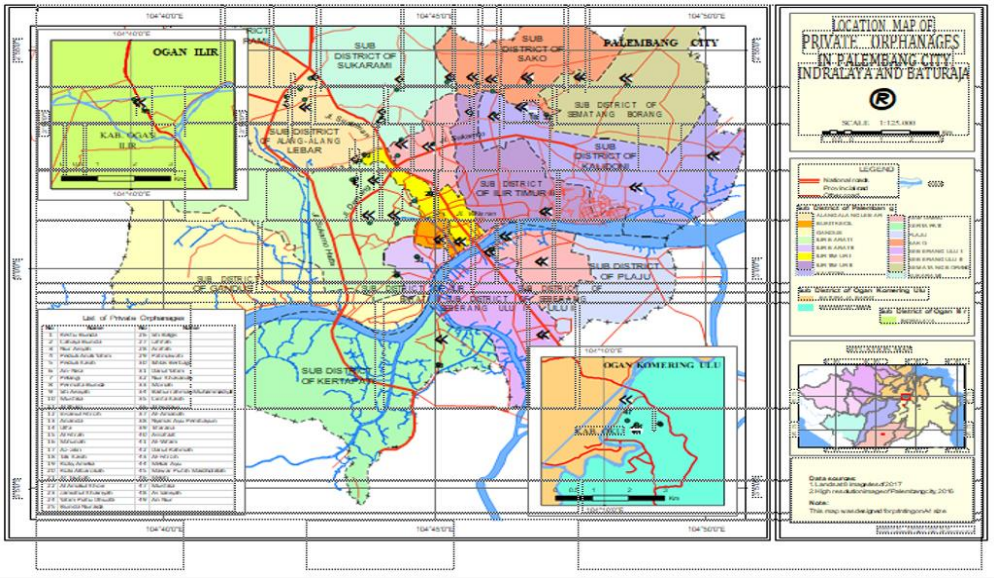

Fig 3: Distribution of *kdr* T917I genotypes in human head lice collected from 12 district in Palembang Subdistrict Within the region Province of South Sumatra, Indonesia

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Fig 3 presents the mean % RAF (average of the RAF for the three kdr-type mutations 100) for each of the 48 collection sites. Of the 48 sites, 32 (95.6%) had lice that had a mean % RAF of 100% (all lice had the three kdr-type mutations; red dots, Fig. 2). Five sites (3.7%) had intermediate mean % RAF ranging for 60–87% (orange dots, Fig. 2). Only a single site had no mutations (0.0 % RAF; green dot, Fig. 2). The overall mean % RAF for all sites was 98.3% with standard deviation of 10.0%.

QS of Collected Lice

In total, 14,281 lice from 479 human subjects from 48 collection sites in 13 subdistrict South Sumatera (Indonesia) for the determination of their RAFs

Table 1: Comparison of human population size versus head lice RAF values

Population size	No. of collection sites	Mean population (6SD)	Total no. of lice analyzed	Avg. no. of lice analyzed (6SD)	Mutation site	Avg. % RAF per allele (6SD)	
High (H)	20	1,042,000 (1,083,00)a	299	14.95e (5.13)	M815I T917I L920F	99%	(0.0)f 100% (0.0)f 99% (0.0)f
Large (L)	45	120,116 (71,845)b	624	13.87e (4.1)	M815I T917I L920F	99%	(3.0)f 100% (2.0)f 99% (2.0)f
Moderate (M)	43	29,749 (12,141)c	607	14.12e (4.14)	M815I T917I L920F	98%	(6.0)f 100% (5.0)f 98% (6.0)f
Low (l)	30	3,548 (2,562)d	395	13.17e (3.31)	M815I T917I L920F	93%	(21.0)f 96% (19.0)f 93% (20.0)f

Abbreviations: RAF, resistance allele frequency; Avg, average number of lice per site.

a–d Mean population sizes are significantly different from one another (unpaired Student's t-test, $P < 0.0001$).

e Average number of lice analyzed per site are not significantly different from one another (unpaired Student's t-test, $P > 0.0d5$).

f Mean % RAF values are not significantly different either in an intrapopulation or an interpopulation comparison (unpaired Student's t-test, $P > 0.05$ [0.5–0.8 range]).

Table 1 There was a total of 1,925 lice analyzed by QS from all sites (1,925/14,281 ¼ 13.55%), resulting in an average of 13.95 lice per site with a standard deviation of 4.10. Randomly selected lice within the most prevalent life stage from all subjects at a site were included for analysis. Females were used for 86 (62%), males for 18 (13%), third instars for 20 (15%), and second for 14 (10%) sites. Average numbers (6SD) of females, males, third and second instars used per site was 14.8 64.6, 12.6 62.3, 12.0 62.2, and 12.5 61.7, respectively. These values were not significantly different from one another (Student's t-test, $P > 0.05$). The average % RAF (6SD) for M815I, T917I, or L920F was 98.1 610.4%, 98.5 69.4%, or 98.3 69.9%, respectively. Given that these values are not significantly different from one another (Student's t-test, $P > 0.05$), we averaged the % RAF for each of the three kdr-type mutations for each collection site in order to graphically display this data set as mean % RAF.

The obtained nucleotide and amino acid sequences were aligned and compared with the published wild-type sequence from the GenBank database using the ClustalW function. The insecticide susceptible *Pediculus humanus capitis* (accession no. AY191156) containing the three amino acid substitution of *kdr* mutation sites was used as the reference sequence. From the multiple nucleotide and amino acid sequence alignment, the homozygous susceptible sequences revealed the presence

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of no substitutions on the base of codon 917, whereas homozygous resistant sequences showed the T917I point mutation due to C ! T substitution leading to Thr (ACA) ! Ile (ATA) mutation. Moreover, all homozygous resistant sequences also contained the L920F point mutation, which showed the nucleotide substitution of the C ! T, leading to Leu (CTT) ! Phe (TTT) mutation. The heterozygous sequences were identified in both non-synonymous mutations of T917I and L920F or showed only a point mutation on codon 920 (Fig 3).

The cloned RS genotype sequences of both wild-type (Clone 1) and mutant (Clone 2) were different at T917I and L920F but the RS genotype sequence from direct sequencing showed double peaks of both C and T at T917I and L920F (Fig 4). The *kdr* sequences were submitted to GenBank under accession numbers MT843902-MT843916. The results obtained on the sample with the SS code: 6, 7, 11, 12, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 29, 32, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44,45,46,47,48; SR code: 1, 2, 13, 25, 26, 28, 31, 34; RR code: 8, 9, 10, 3, 5, 27, 30, 33 (Fig 3). Molecular analysis in Fig. 3 shows that the SR code describes DNA experiencing mutations originating from the subdistrict namely Ilir Timur 1, Seberang Ulu 1, Kalidoni, Ilir Barat 1, Ilir Barat 1 and the RR code describes DNA experiencing mutations and resistance originating from the subdistrict namely only Ilir Timur 1, Ilir Timur 2, Seberang Ulu 1, Kalidoni, Ilir Barat 1, Ilir Barat 2.

DISCUSSION

Human head lice collected from children living in twelve orphanages in different subdistricts of Palembang city were screened for knockdown resistance (*kdr)* mutations against the pediculicide, permethrin. The overall frequency of the *kdr* mutation was 0.31, which is considerably modest compared to previous reports of a high prevalence of the *kdr* mutation in the US, Canada, and Europe (22, 24). The lower prevalence of the *kdr* mutation among head louse populations in Thailand could be due to infrequent usage of pyrethroid in rural areas. The number of infestations in rural areas tends to be higher than in urban areas with less access to certain pediculicides (5,6). Consequently, instead of the contemporary scientific drug store pediculicides, traditional methods are the mainstay treatment to eliminate the louse. Folk remedies are based on the use of herbs like custard apple leaves, plant-based herbal shampoo, occlusion techniques as well as anti-louse medical devices (33, 41). In urban areas, pediculosis cases are more properly managed with pediculicides, as they are more accessible at either over-the-counter pharmacies or dermatology clinics. Most of the prescribed pediculicides in Indonesia contain permethrin and pediculicides other than permethrin are not widely available in Palembang city. Regarding the overall possession of *kdr* alleles among the collected head lice, 156 of the 260 lice were homozygous susceptible which is the predominant genotype, 58 lice were homozygous resistant (RR), and 46 lice were the heterozygous genotype (RS). Therefore, the lice populations accounted for 40% of either one or two *kdr* T917I mutations. Despite the lower prevalence of the *kdr* mutation of head lice in our study in Palembang, one other study conducted in 2010 documented no resistant strains of human head lice in Palembang (19). The resistant strain of human head lice to pyrethroid was first reported in our study conducted several years after the aforementioned study. To date, despite a relatively lower incidence of resistance in comparison with countries in America or Europe, accumulating evidence suggests that the resistance issue

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should not be overlooked. The possible explanations for emerging pyrethroid resistance in human head lice could be the increasing use of insecticides over the past few years, new surveys covering a larger area with larger sample sizes compared to the previous study, as well as the inadequate or misuse of pediculicides leading to treatment failure, recurrence, or reinfection, especially in high prevalence rural areas of the country.

To determine pyrethroid resistance in lice, both biochemical and molecular techniques can be applied (26,36). The molecular approach, PCR-RFLP, was adopted in the present study as it is low cost, less time-consuming, and reproducible. Previous studies have demonstrated that particular point mutations associated with permethrin resistance in the *VGSC* gene of human head lice were an M815I-T929I-L932F *kdr* mutation (26,32,36,43). The belief that these three mutations coexist has led to most recently published papers choosing to study the T929I mutation. Yoon et al. (2) established the presence of the T929I and L932F mutation (T917I and L920F, respectively, in head louse amino acid sequence) in permethrin resistance head lice from children in South Florida and like our study, identified two locations of the point mutation.

To confirm the reliability of RFLP methods for a particular amino acid substitution, we randomly selected samples from three genotypes to perform DNA sequencing, showing that DNA sequencing results directly correlated with the pattern of RFLP detected bands in all three genotypes. The chromatogram of the heterozygous genotype simultaneously generated double peaks (two colours) and half the height of the signal intensity at the T917I and L920F point mutations. The chromatogram of the cloned PCR products of direct sequencing produced two distinct genomic DNA including wild-type and mutant patterns, confirming the genotypic heterozygosity and demonstrating two distinguishing DNA variations. Accordingly, these findings emphasised that PCR-RFLP is a promising technique for knockdown resistance detection in head lice because of its accuracy in identifying particular point mutations. To determine the genotype frequencies of the collected head lice population, we used the exact test of H-W equilibrium and found that five localities, except that of eastern Thailand differed from expectations. Additionally, those five localities also had a positive inbreeding coefficient value ($Fis > 0$) which could be interpreted as homozygous excess, an indicator of inbreeding or in fixation of mutant alleles. In contrast, the first study of pyrethroid resistance in head lice from Honduras by Larkin et al. (17) using RFLP techniques demonstrated that most head lice had an excess of heterozygotes of *kdr*-type mutations which indicates the active selective pressure among head lice.

In terms of the variation of permethrin resistance in human head lice among studies, it is believed to mainly stem from the varying prevalence of pediculosis and the frequency of pediculicides use in different countries or continents of the world. However, the overall prevalence has been increasing annually (13,22,24,26,30,38,44). The *in vitro* mortality bioassay data showed a high percentage of agreement between the phenotypic (resistant) and genotypic (homozygous resistance) determinations within the collected South Florida population (42). The findings from this study emphasised the recessive inheritance of the *kdr* mutation. There was no cross-resistance between malathion and permethrin and those permethrin-resistant head lice were still susceptible to malathion, thereby supporting the use of malathion and other pediculicides for treatment. The

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first detection of *kdr*-resistant head lice in Thailand may be due to repeated episodes of pediculosis, especially in elementary school children (with improper prevention) leading to continuous exposure to pediculicides or in some cases, inadequate treatment. This could result in permethrin-resistant head lice among samples of head lice collected from elementary school children in various areas of Thailand. To help reduce the unnecessary permethrin or other pediculicide uses, the public health approach for community head lice infestation and school health including educational programmes for all related groups of people, for example, parents, school staff, and local healthcare professionals should be done regularly (41). In some areas with a high prevalence of pediculosis, pediculicide-free treatment choices, such as occlusive agents, herbal medicine, and anti-louse devices (heated air, suction, or electronic combs) (33,41). are preferable due to their better safety profile, greater availability and no risk of developing resistance. However, for herbal agents, the efficacy and safety are not yet well-established (33,43,44). Attention has turned towards medical devices rather than medicinal products with less control and regulation. Nonetheless, in some countries, permethrin remains the first-line medication for the treatment of pediculosis unless permethrin resistance is suspected in the community (33,40,43).

This study has some limitations. The number of collected human head lice in some areas was quite lowand might not represent the real situation of pyrethroid resistance in those particular areas. Molecular techniques were used to demonstrate genotypic resistance which may not infer the phenotypic level of pyrethroid resistance. Moreover, the head louse samples used in this study were collected for the previous Sunantaraporn et al. study (4) whichmight not provide an up-to-date status of head lice resistance to permethrin in orphanages in the Palembang Subdistrict within South Sumatra.

In summary, this is the first study to detect permethrin resistance in human head lice collected from orphanages of the Palembang Subdistrict within South Sumatra. PCR-RFLP can be used to evaluate the presence of *kdr* mutations in the head louse, revealing three different genotypes in the head lice collected from the orphanages located in the Palembang Subdistrict within South Sumatra. More samples are required, particularly from the eastern area, for future studies to provide a better understanding of permethrin resistance among primary school children in the Palembang Subdistrict within South Sumatra. Further studies should also focus on the correlation between genotypes and phenotypes of human head lice permethrin resistance.

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