The frequencies of knockdown resistance mutations in phlebotomine sandflies under different degrees of indoor residual spraying

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(Received: 11 May 2021; Accepted: 19 August 2021)

Abstract: The emergence of pyrethroid resistance in Phlebotomus sandflies is an urgent issue for vector control using indoor residual spraying (IRS). Two amino acid substitutions at codon 1014 (L1014F and L1014S) in the voltage-gated sodium channel (VGSC) gene have been reported in Phlebotomus argentipes, a major vector of visceral leishmaniasis (VL). Known as “knockdown resistance (kdr),” these substitutions confer pyrethroid resistance in various insect species. The frequency of the VGSC mutant allele was investigated in Phlebotomus and Sergentomyia species at two different IRS regimes: “long-term treated,” 12 rounds for seven years in Mymensingh, Bangladesh; “short-term treated,” four rounds for two years in Pabna, Bangladesh. In Mymensingh, the L1014F/S allele frequency was 100% in P. argentipes and 98% in S. babu babu. In Pabna, the frequency was 41% in P. argentipes. At other kdr sites (codons 1011, 1016, and 1020), the genotypes of all specimens in Bangladesh were wild-type homozygotes. This study showed that a long and frequent exposure to IRS is crucial for the development of genetic mutations in VGSCs, a higher kdr frequency, and pyrethroid resistance in Phlebotomus.

Key words: Bangladesh, kdr, Phlebotomus argentipes, pyrethroid resistance, Sergentomyia babu babu, voltage-gated sodium channel

INTRODUCTION

Phlebotomine sandflies are a subfamily of the Psychodidae (Order: Diptera) family. Their body sizes are approximately 2–3 mm long, and their habitats range from rainforests to deserts between the latitudes 50°N and 40°S (Young and Perkins, 1984; Lane, 1993). Phlebotomine sandflies are divided into six genera (Phlebotomus, Sergentomyia, and Chinius distributed in the Old World, and Lutzomyia, Brumptomyia, and Warileya distributed in the New World), with the total number of species exceeding 800 (Lane, 1993; Young and Duncan, 1994). Some sandfly species are known to be vectors of several infectious diseases, including leishmaniasis (caused by Leishmania protozoans), bartonellosis (caused by Bartonella bacilliformis Strong), Channipura virus encephalitis (caused by the Channipura virus), and diseases caused by Phlebovirus (Maroli et al., 2013). The main genera of phlebotomine sandflies known to transmit these pathogens are Phlebotomus, Lutzomyia, and Sergentomyia (Maroli et al., 2013; Damle et al., 2018).

Of all sandfly-borne diseases, leishmaniasis is the most epidemiologically important disease to control due to the large number of patients involved. The disease is regarded as one of the neglected tropical diseases (NTDs) (WHO, 2010a), and the effort for its elimination led by the World Health Organization has continued. There are three different forms of leishmaniasis: visceral leishmaniasis (VL), cutaneous...
leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL). The VL form is the most severe and fatal if it is left untreated. In 2012, the annual number of VL cases was estimated to be around 200,000 to 400,000 worldwide, and three countries, Bangladesh, India, and Nepal on the Indian subcontinent (ISC), account for 80% of the cases (Alvar et al., 2012). In these countries, *Phlebotomus argentipes* Annandale and Brunetti is considered as the vector (WHO, 2010b; Swaminath et al., 2006), and the main protozoan parasite is *Leishmania donovani* Laveran and Mesnil (Thakur et al., 2018). In contrast, the potential ability to transmit *Leishmania* protozoans of *Sergentomyia* has been suggested by recent studies that detected *Leishmania* DNA from several *Sergentomyia* species in the wild (Berdjane-Brouk et al., 2012, Ayari et al., 2016, Pereira et al., 2017, González et al., 2020).

Vector control using insecticides plays an important role in the prevention and elimination of diseases. In the ISC, the Kala-Azar Elimination Programme (KAEP) was launched in 2005 to eliminate VL. The KAEP’s regional strategy contains integrated vector management using insecticides (Kazi, 2011) in impregnated bed nets and indoor residual spraying (IRS), primarily using pyrethroids. Bangladesh, India, and Nepal participated in the program and began cooperating to reduce the number of VL cases in the ISC (WHO, 2005). However, the emergence and existence of insecticide resistance has become a serious problem. In India, resistance to deltamethrin in *P. argentipes* was reported from Pondicherry (Amalraj et al., 1999). Deltamethrin is a commonly used pyrethroid in the ISC. In Nepal, *P. argentipes* from three VL-endemic districts showed signs of resistance to two pyrethroids, alpha-cypermethrin and deltamethrin, which are used for IRS in the ISC (Chowdhury et al., 2018a).

One of the major mechanisms of pyrethroid resistance among arthropods is knockdown resistance (*kdr*) mutations in the voltage-gated sodium channel (VGSC) gene. This study aimed to evaluate the continuous effect of IRS on the *kdr* allele frequency in phlebotomine sandflies in Bangladesh. There, vector control targeting sandflies began in 2012 (Chowdhury et al., 2018b). The first round of IRS was conducted in VL-endemic districts using deltamethrin 5WP (wettable powder) (25 mg/m²). For sustainable vector control, monitoring the emergence and spread of *kdr* alleles in sandfly populations is essential. *Phlebotomus* and *Sergentomyia* sandflies under different IRS situations (Mymensingh and Pabna districts in Bangladesh) were used to compare the *kdr* allele frequency. This is the first study that compared the impact of IRS exposure on the *kdr* allele frequency in phlebotomine sandflies, and the first report of the presence of *kdr* mutations in *Sergentomyia*.

**Materials and Methods**

1. Collection of sandflies

*“Long-term” IRS treatment (Site: Mymensingh, Bangladesh)*

Collection was conducted in Magurjora village, Harirampur Union, Trishal sub-district (Upazila), Mymensingh District, Mymensingh Division, Bangladesh for two days on October 24 and 25, 2018 (24.5212°N, 90.4124°E) (Fig. 1). In Mymensingh, which is the most endemic area for VL in Bangladesh, IRS has been conducted since 2012. *Phlebotomus argentipes* and *Sergentomyia babu babu* Annandale were selected for this study. *Sergentomyia babu babu* was the only *Sergentomyia* species collected. Sandflies were collected using 10 Centers for Disease Control and Prevention (CDC) miniature light traps during the night. The traps were placed in and around human dwellings and cattle sheds. The captured sandflies were transferred to 80% ethanol and stored at room temperature for morphological identification.

*“Short-term” IRS treatment (Site: Pabna, Bangladesh)*

Collection was conducted in Chhaikhola Union, Chatmohar sub-district (Upazila), Pabna District, Rajshahi Division, Bangladesh for three days from March 8 to 10, 2014 (24.3109°N, 89.3091°E) (Fig. 1). In Pabna, two IRS rounds were conducted once a year in 2012 and 2013. The method used to collect sandflies was the same as that used in Mymensingh. In total, 26 CDC miniature light traps were used during the three days.

**Japanese sandflies as reference**

Sandflies in Japan were used as reference, which were in the area IRS had never been conducted. Collection was conducted in five Prefectures in Japan: Niigata (38.0697°N, 138.3791°E), Gunma (36.7742°N, 138.9425°E), Tokyo (35.8161°N, 139.0882°E), Shizuoka (35.0683°N, 139.0162°E), and Nagasaki (33.0608°N, 129.9401°E). In Japan, a sandfly-targeted chemical control using insecticides has never been conducted. Sample collection was conducted using sticky paper traps (Sanjoba et al., 2017) or CDC miniature light traps for one day at each site. The sticky paper traps were made by coating A4-sized sheets of paper with castor oil and placed in the bush or tussock. The captured sandflies were transferred to 80% ethanol and stored at room temperature.

2. Identification of species

The heads and abdominal segments of the specimens were cut off from their bodies and transferred to labeled slides containing Swan solution. The remaining bodies were stored in 99.9% ethanol. Identification of species was conducted using
published keys and descriptions (Lewis, 1978; Lewis, 1982), based on the features of the cibariums, pharynx, and spermathecae in females, and genitalia, coxites, and styles in males. These features were observed under BX51 and SZ61 microscopes (Olympus Co., Tokyo, Japan) and recorded using a DP73 microscope camera (Olympus Co.). After identification, P. argentipes and S. babu babu from Mymensingh, P. argentipes from Pabna, and all specimens from Japan were used for further DNA extraction.

3. DNA extraction and VGSC codon genotyping

The DNA of each specimen was extracted individually. The DNA of Bangladeshi specimens was extracted using MagExtractor™-Genome- (TOYOBO Co., Ltd., Osaka, Japan). Before the extraction, a 10 µL mixture containing 1 µL Proteinase K (1,354 units/mL, Sigma-Aldrich Co. LLC., St. Louis, MO, USA), 0.5% SDS, 2 mM CaCl₂, 8 mM Tris-HCl, and 8 mM EDTA was added to each specimen in a well of a PCR plate and homogenized using TissueLyser II (QIAGEN N. V., Venlo, Netherlands) at 30 Hz for 3 min with a zirconia bead (2.3 mm) (BMS, Tokyo, Japan). After overnight incubation at 55°C, MagExtractor™ was used and the DNA was finally eluted into 25 µL Low TE (10 mM Tris-HCl and 0.1 mM EDTA). The DNA of Japanese specimens was extracted via alkali extraction. Zirconia beads and 10 µL of 0.2 M NaOH were added to each specimen and homogenized using TissueLyser II at 30 Hz for 3 min. After a 10-min incubation at 70°C, a 40 µL mixture containing 22.5 mM Tris-HCl and 0.3125 mM EDTA was added for neutralization. All extracted DNA samples were stored at −20°C until further analysis.

PCR amplification of the partial VGSC gene fragments was performed to evaluate the existence of kdr mutations. The primers used for P. argentipes contain Affinity Plus bases (IDT, Inc., Coralville, IA, USA) that increase hybridization specificity only for the P. argentipes sequence. The primers used were PargVgscF (5’-TGG GAG AAT GGT TGC TGA TAG ACT TGA-3’) and PargVgscR (5’-GTCTGCGATGTGGTGCTGATGACTTAAGTGA-3’), where the bases that follow “+” are Affinity Plus bases. The primers used for Sergentomyia were Vssc8f (5’-AAT GTG GGA TTG CAT GCT G-3’) and Vssc1br (5’-CGT ATC ATT GTC TGCTG-3’), which were as previously described (Gomes et al., 2017) and are commonly used for phlebotomine sandfly kdr analysis. Both primers amplify the para VGSC gene fragments, including codon 1014, a previously reported sandfly kdr site (Gomes et al., 2017, Fotakis et al., 2018, Sardar et al., 2018, Pathirage et al., 2020), as well as codons 1011, 1016, and 1020, which are known kdr sites in other insects and targeted codons in previous research (Gomes et al., 2017, Fotakis et al., 2018, Sardar et al., 2018, Fotakis et al., 2020, Pathirage et al., 2020). Amplification was conducted separately, where each
25 μL reaction mixture contained 12.5 μL of 2× PCR buffer for KOD FX (TOYOBO Co., Ltd.), 5 μL of 2 mM dNTPs, 0.75 μL of 10 μM forward and reverse primers, 0.5 μL of KOD-FX DNA polymerase stock solution (1 U/μL) (TOYOBO Co., Ltd.), and 1 μL of genomic DNA. The thermocycling conditions consisted of an initial denaturation step of 5 min at 95°C, followed by 38 cycles of 96°C for 30 s, 56°C for 30 s, and 68°C for 30 s, and a final extension step at 68°C for 5 min. The PCR product size, quality, and quantity were analyzed via microchip electrophoresis with MultiNA (Shimadzu Co., Kyoto, Japan). The products were cleaned using ExoSAP-IT (Thermo Fisher Scientific Inc., Waltham, MA, USA). The BrilliantDye Terminator v1.1 Cycle Sequencing Kit (NimaGen B. V., Lagelandseweg, Netherlands) was used for the cycle sequencing of the post-clean-up products. The fluorescence in the cycle sequencing products was read using a 3130 Genetic Analyzer (Thermo Fisher Scientific Inc.) to determine the nucleotide sequences. The mutations were analyzed using BioEdit Sequence Alignment Editor v7.0.5 (Hall, 1999). The codon numbering was based on the para VGSC sequence of the housefly Musca domestica (GenBank accession number: X96668).

4. Alignment of coding sequence (CDS) haplotypes

The CDS haplotypes in the VGSC gene fragments were aligned using ClustalW 2.1 (Larkin et al., 2007) with the sequences from P. argenteipes in Bihar, India from previous research (GenBank accession numbers: KY114615-18).

RESULTS

1. The frequency of kdr alleles at three IRS situations

“Long-term” IRS treatment (Site: Mymensingh, Bangladesh)

In total, the number of collected sandflies was 100, of which 65 were P. argenteipes and 35 were S. babu babu. All specimens were used for the genotyping, and 65 P. argenteipes and 24 S. babu babu were successfully genotyped for the VGSC gene (Table 1). In P. argenteipes, three different alleles, TCA (L1014S), TTT (L1014F), and TTC (L1014F), were detected at codon 1014. The wild-type (susceptible) allele TTA was not found in any of the specimens. The allele frequencies of TCA, TTT, and TTC were 29.2% (38/130), 65.4% (85/130), and 5.38% (7/130), respectively (Table 2). In S. babu babu, the allele TTA, which encodes leucine and is considered wild-type, was detected in addition to two alternative alleles, TCA (L1014S) and TTT (L1014F).

### Table 1. The number of genotyped male/female specimens from each site of three IRS periods.

| Period of IRS | Nation | Place          | Geographical coordinate | Type of traps | Species      | Male | Female | Total |
|---------------|--------|----------------|-------------------------|---------------|-------------|------|--------|-------|
| 7 years       | Bangladesh | Mymensingh | 24.5212, 90.4124 | Light trap   | P. argenteipes | 43   | 22     | 65    |
|               |         |              |                         |               | S. babu babu | 5    | 19     | 24    |
| 2 years       | Bangladesh | Pabna        | 24.3109, 89.3091 | Light trap | P. argenteipes | 56   | 63     | 119   |
| Never         | Japan    | Niigata      | 38.0697, 138.3791 | Light trap   | S. squamirostris | 12   | 21     | 33    |
|               |         | Gunma        | 36.7742, 138.9425 | Light trap | ''          | 18   | 22     | 40    |
|               |         | Tokyo        | 35.8161, 139.0882 | Light trap | ''          | 0    | 5      | 5     |
|               |         | Shizuoka     | 35.0683, 139.0162 | Light trap | ''          | 16   | 0      | 16    |
|               |         | Nagasaki     | 33.0608, 129.9401 | Light trap | ''          | 22   | 20     | 42    |
|               |         |              |                         |               | Total       | 68   | 68     | 136   |

### Table 2. Genotype and allele frequencies at codon 1014 in phlebotomine sandflies of three IRS periods.

| Period of IRS | Species | Country | Place          | Number of individuals | Number of alleles |
|---------------|---------|---------|----------------|-----------------------|------------------|
|               |         |         |                |                       |                  |
| 7 years       | P. argenteipes | Bangladesh | Mymensingh | 0 (0) | Leu/Leu: 0 (0) |
|               | S. babu babu | Bangladesh | Mymensingh | 0 (0) | Ser/Ser: 0 (0) |
| 2 years       | P. argenteipes | Bangladesh | Pabna | 52 (3.7) | Ser/Ser: 20 (10) |
| Never         | S. squamirostris | Japan | 5 Prefectures | 136 (100) | Ser/Ser: 0 (0) |

Leu=leucine; Ser=serine; Phe=phenylalanine. Values in brackets are percentage within each group.
at codon 1014. The allele frequencies of TTA, TCA, and TTT were 2.08% (1/48), 60.4% (29/48), and 37.5% (18/48), respectively (Table 2). At codons 1011, 1016, and 1020, all genotypes were wild-type homozygous (I1011, V1016, and F1020) in both species. The GenBank accession numbers of the partial VGSC gene sequence obtained from *P. argentipes* and *S. babu babu* in Mymensingh were LC635948–LC635951 and LC636133–LC636135, respectively.

"Short-term" IRS treatment (Site: Pabna, Bangladesh) In total, the number of collected sandflies was 280, of which 247 were *P. argentipes* and the rest was *S. babu babu* in Mymensingh. Out of 247 *P. argentipes* specimens, 120 were used for genotyping and 119 of them were successfully genotyped (Table 1). Only the wild-type allele was detected at codons 1011, 1014, 1016, and 1020 (Table 2). The GenBank accession number of a typical sequence of the partial VGSC gene obtained from *S. squamirostris* in Japan was LC636136.

Japanese sandflies as reference All sandflies collected in Japan were morphologically identified as *Sergentomyia squamirostris* Newstead. Of all *S. squamirostris* specimens, 140 were used for genotyping and 136 of them (33 from Niigata, 40 from Gunma, five from Tokyo, 16 from Shizuoka, and 42 from Nagasaki) were successfully genotyped (Table 1). Only the wild-type allele was detected at codons 1011, 1014, 1016, and 1020 (Table 2). The GenBank accession number of a typical sequence of the partial VGSC gene obtained from *S. squamirostris* in Japan was LC636136.

2. Variations in the phlebotomine partial VGSC sequences

The VGSC gene fragment investigated in this study contained exons 19 and 20. Exon 19 contains codons 1011 and 1014, and exon 20 contains codons 1016 and 1020. Fig. 2 shows that variations were found only at codon 1014 among *P. argentipes* at three sites (Bihar, Mymensingh, and Pabna). The nucleotide sequences of the *Sergentomyia* species were different from those of *P. argentipes*, although the translated amino acids were identical.
**Discussion**

VGSCs play an essential role in the initiation and propagation of action potentials on nerve cells. Pyrethroids inhibit the proper function of VGSCs and cause immediate paralysis and death in insects. Several amino acid substitutions on VGSCs are known to make them less sensitive to pyrethroids. These substitutions, known as knockdown resistance (kdr) mutations, are seen in many medically and agriculturally important insect pests (Soderlund and Knipple, 2003; Rinkevich et al., 2013). Although several kdr substitutions are known to date, amino acid mutations at the L1014 site (using *M. domestica* Linnaeus codon numbering) of the VGSC are commonly observed in a wide range of insect species, such as German cockroaches, houseflies, and *Anopheles* mosquitoes (Miyazaki et al., 1996; Rinkevich et al., 2013). As for sandflies, L1014F/S substitutions were first reported in *P. argentipes* from Bihar, India in 2017 (Gomes et al., 2017). That study reported that both the L1014F and L1014S alleles are genetically associated with DDT and pyrethroid resistance. Subsequent research in 2018 also reported the existence of L1014F/S alleles and their association with pyrethroid resistance in *P. argentipes* from West Bengal, India (Sardar et al., 2018). A more recent study showed that *P. argentipes* from Sri Lanka also had L1014F/S alleles (Pathirage et al., 2020). This study revealed that sandflies in Bangladesh also had kdr alleles at codon 1014.

Our study site, Mymensingh, is the most endemic area for VL in Bangladesh, and *P. argentipes* is the dominant species (Özbel Y et al., 2016). In Mymensingh, IRS had been conducted 12 times from 2012 to 2018 before the collection time of this study. In Mymensingh, the 95% confidence interval (CI) of kdr allele frequency was estimated to be 97.2–100% in *P. argentipes*, which was higher than in Pabna (95% CI, 34.5–47.3%). Pabna is not as endemic for VL in Bangladesh, and *P. argentipes* is desired. In the Japanese sites where IRS was never conducted, all *S. squamirostris* specimens had wild-type alleles at codon 1014. This result suggests that kdr was not selected in *S. squamirostris* because insecticide selection had hardly been applied to this species.

The allele frequency of L1014F (70.8%) was higher than that of L1014S (29.2%) in *P. argentipes* from Mymensingh. In contrast, the allele frequency of L1014S was higher than that of L1014F in *P. argentipes* from Pabna and *S. babu* from Mymensingh. Previous studies have reported a higher allele frequency of L1014S compared to L1014F in India, whereas the opposite was true in Sri Lanka (Gomes et al., 2017, Sardar et al., 2018, Pathirage et al., 2020). Generally, the frequency of resistance alleles in a population is affected by selective advantage, initial frequency, and migration from other populations. In an electrophysiological study using *Drosophila* VGSCs, L1014F was suspected to confer stronger resistance against both permethrin and deltamethrin than L1014S did (Burton et al., 2011). If there is little
difference in fitness costs between these two alleles in sandflies, it is expected that the L1014F frequency of *S. babu babu* will increase in the future due to selection by pyrethroids. However, further studies are needed to evaluate the biological nature of each allele in detail.

Continuous surveillance for insecticide resistance is essential for selecting the most appropriate measure for vector management. In the Old World, both *Phlebotomus* and *Sergentomyia* sandflies are medically important. Therefore, monitoring pyrethroid resistance in both genera is needed. In the ISC, pyrethroids are considered to be more effective than DDT because *Phlebotomus* in VL-endemic areas are highly resistant to DDT (Dinesh et al., 2010, Singh et al., 2012, Singh and Kumar, 2015, Dhiman and Yadav, 2016). However, given the high frequency of *kdr* in *P. argentipes*, alternative insecticides to pyrethroids may need to be considered. Since a practical method to breed sandflies for insecticidal tests has not yet been established, a genetic analysis of field-collected samples could be effective in monitoring their resistance status. In addition, elucidating the existence of other resistance-conferring mutations in the VGSC, as well as the involvement of other resistance-related genes (e.g., detoxification enzymes), are also important in future studies to fully understand the mechanisms of pyrethroid resistance in this insect group.

**Acknowledgements**

The authors appreciate the support given by Mr. Md. Abdus Salam, Trishal Health Complex, and Ms. Sohana Asma during the sandfly collection period in Magurjora village. This work was part of a project entitled Research and Development of Prevention and Diagnosis for Neglected Tropical Diseases, supported by JST/JICA and the Science and Technology Research Partnership for Sustainable Development, and supported by JSPS KAKENHI (Multi-year Found) Grant number 19kk0274. This research was also partially supported by the Research Program on Emerging and Re-emerging Infectious Diseases from Japan Agency for Medical Research and Development (AMED) under Grant Numbers JP20wm0225007, JP20fk0108067, JP18fk0108035, and JP20fk0108139, Oshimo Foundation and JSPS KAKENHI Grant Number JP16K07476.

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