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ABSTRACT

Through its unique mode of action, ivermectin represents a relatively new and very promising tool to fight against human lice, especially in cases of resistance to commonly used pediculicides. However, ivermectin resistance in the field has already begun to be reported. Therefore, understanding the mechanisms involved is a key step in delaying and tackling this phenomenon.

In this study, field head lice with confirmed clinical resistance to ivermectin in rural human populations from Senegal were subjected to genetic analysis targeting the GluCl gene, the primary target of ivermectin known to be involved in resistance. Through DNA-polymorphism analysis, three relevant non-synonymous mutations in GluCl which were found only in ivermectin-resistant head lice (76 head lice tested), were identified. The A251V mutation found in the TM3 transmembrane domain was the most prevalent (allelic frequency of 0.33), followed by the S46P mutation (0.28) located at the N-terminal extracellular domain. The H272R was in the M3–M4 linker transmembrane region of GluCl and has shown the lowest frequency (0.18). Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) diagnostic assays were also developed for their accurate genotyping.

Our study is the first to report the presence of GluCl point mutations in clinical ivermectin-resistant head lice occurring in rural human populations of Senegal.

1. Introduction

The head lice, *Pediculus humanus capitis*, are obligate blood-sucking parasites that live exclusively in the scalp region of humans [1,2]. They represent one of the most prevalent parasitic infestations with major economic and social concerns throughout the world [3]. Body lice are the main vectors of at least three human pathogens: *Rickettsia prowazekii*, the agent of epidemic typhus; *Bartonella quintana*, the agent of trench fever; and *Borrelia recurrentis*, the agent of relapsing fever. Currently, head lice are not considered to be vectors for human pathogens. However, the DNA of several pathogenic bacteria, such as *B. quintana, B. recurrentis*, and *Yersinia pestis,* has already been detected in head lice [4–7]. Besides, experimentally infected head lice are capable of acquiring, maintaining and transmitting *R. prowazekii* and *B. quintana* [8,9]. Overall, these data support the suggestion that head lice have the potential to be vectors of pathogens under optimal epidemiological conditions.

Treatment of pediculosis relies widely on the use of topical insecticides, with a neurotoxic mode of action, such as topical pyrethroids or malathion [10]. Unfortunately, the extensive use of these insecticides has led to the emergence and spread of resistant louse populations all over the world [1,10,11]. This resistance development has prompted research for new treatment strategies such as ivermectin. This drug gained heightened attention as it exhibited higher lethal capacity against insects with only small doses of product [12]. Moreover, the mechanism of action of ivermectin differs from all classes of pediculicides used to treat lice providing opportunities to counter the existing resistance [11,13].

Ivermectin is a macrocyclic lactone, a multifaceted ‘wonder’ drug that has a broad spectrum of activity. It acts robustly against a wide variety of nematodes and arthropods [12,14]. Its unexpected potential as an antibacterial, antiviral, and anti-cancer agent is also particularly valuable in improving global public health [12,15,16]. Ivermectin is already deployed and commercialized to treat human lice infestation. Several reports indicated that both orally

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Table 1
Primer sequences used in this study.

Purpose	Primer name	Primer sequences (5′–3′)	Product size (bp)
GluCl	6079F	CAATTAATTCGACGGATTCAG	1722
	7800R	GATTGATTTACCAACGACGGC	
Sequencing of 1722-bp GluCl	7157F	AGTGACAACATTACTCACAA	
	6574F	GCTCACTTCGAATGGCCAGTTG	
	6686R	CCATCCCTACGACCATCAAATT	
	7287R	CTAACAAAGCTCCAAATACGAAC	
PCR-RFLP	5794F	AACCAAACGGATAAAACCGATG	865 (for S46P genotyping) (AgsI: 550+315)*
	6658R	CCGACCAAAAGCTTTAAATTAT	
	6833F	AAGTTGGCGATCCCGTTCAAG	919 (for H272R genotyping) (NsiI: 379+540)*
	7750R	CAATCGAATTAATTATCTTCCGT	
	7203F	GCATCATTGCCACCGGTA	547 (for A251V genotyping) (HhaI: 440+107)*
	7750R	CAATCGAATTAATTATCTTCCGT	

bp, base pairs; PCR-RFLP, polymerase chain reaction restriction fragment length polymorphism.
* Length of fragment obtained after digestion with the corresponding restriction enzyme endonuclease.

and topically formulations were highly effective in controlling lice infestations [11,17,18]. The mode of action of ivermectin makes it safer for use on vertebrates, including humans, as it targets the glutamate-gate chloride (GluCl) channels present in invertebrates [14,19]. Such channels do not exist in vertebrates and the drug has a low affinity for other vertebrates' ligand-gated chloride channels [14]. GluCl channels contribute extensively to invertebrate nervous system function, including modulating locomotion, regulating feeding, and mediating sensory inputs [20,21]. Thus, the modification of channel function by ivermectin results in paralysis and death [21].

GluCl channels are pentamers belonging to the Cys-loop receptor superfamily, constituting five subunits organized to form a chloride-permeable pore at the center [22,23]. Each subunit has an extracellular N-terminal domain, which contributes to the glutamate-binding site, and four membrane-bound helices (M1–M4) that constitute a channel domain [24,24,25]. The ivermectin-binding site is in the channel domain, lying between M3 and M1 of two adjacent subunits. Ivermectin also makes contact with M2 helix, which lines the pore of the ion channel, through its disaccharide moiety and the M2–M3 loop [21].

As with other insecticides, ivermectin is subject to selection pressures that have led to resistance development. Currently, this resistance has been demonstrated in many arthropods and is an increasing problem for their control. Therefore, resolving the mechanisms of resistance is an important research priority [21,26,27]. Target-site insensitivity is one of the principal mechanisms by which insect pests acquire resistance to insecticides [22]. Indeed, several studies have shown that mutations in GluCl are associated with ivermectin resistance in several arthropods and worms [26–29].

Recently, Diatta et al. (2016) reported the first field-evolved ivermectin resistance in head lice from Senegal [30]. These lice were recovered from two females after ivermectin treatment. This study aimed to investigate the presence of polymorphisms in the ivermectin target site GluCl, known to be involved in ivermectin resistance, of these lice.

2. Materials and methods

2.1. Study design and louse sampling

Samples genotyped in the present study were collected in November 2015 in Dielmo, a village located in the Sine-Saloum region of Senegal, during an ivermectin oral treatment trial. This trial aimed to confirm previous suspicions of ivermectin resistance observed during a first clinical trial conducted in 2014 in the same village [30]. The full details of the study were described by Diatta et al. [30]. Briefly, ivermectin (400 µg/kg of body weight) was orally administered in one intake to treat eight females, six with suspected resistance or re-infestation observed during the first trial and two in whom no resistance or re-infestation phenomena were observed. After 24 h of ivermectin treatment, two cases (2/8; 25%) of clinical ivermectin resistance (treatment failure) were observed from which six live lice were recovered. Head lice from all these females (6 live lice and 30 dead lice after ivermectin treatment) as well as lice collected from the first trial, were sent to our laboratory in Marseille (France) to investigate the possible mechanisms involved in ivermectin resistance. The Orlando (Culpepper) reference strain of body lice maintained on rabbits, never exposed to ivermectin, was used as a control wild type.

2.2. Amplification, cloning, and sequencing of GluCl gene

Genomic DNA (gDNA) was extracted from louse specimens as described previously [4]. The gDNA sequence of the GluCl gene of *P. humanus* (GenBank ID: PHUM451790-RA, DS235786) has 7740 base pairs (bp), consisting of 7 exons and 6 introns. The GluCl fragment (~1722 bp) encompassing all exons, except the first exon which is composed of 3 bp, was polymerase chain reaction (PCR) -amplified using a set of primer pairs listed in Table 1. The amplification was performed from a DNA pool of the six head lice with suspected ivermectin resistance and the laboratory susceptible body lice strain to perform DNA polymorphism analysis.

All PCRs were performed using a Peltier PTC-200 model thermal cycler (MJ Research Inc., Watertown, MA, USA) with the Hotstar Taq-polymerase (Qiagen, Hilden, Germany). The purified PCR products were ligated into a pGEMT-easy vector (Promega, Madison, WI) and transformed into JM109 Competent Cells. The plasmid inserts were then PCR amplified using a vector-specific primer set (M13 forward and reverse primers) and subjected to sequencing using the Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with an ABI automated sequencer (Applied Biosystems). The electropherograms were assembled and edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia). Analysis of the nucleotide and amino acid sequences was conducted using the ClustalW2 computer program (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

2.3. Molecular diagnostic assays based on PCR-RFLP for GluCl mutations genotyping

Following the identification of mutations potentially associated with ivermectin resistance, three PCR-RFLP diagnostic assays were designed for their accurate detections. The appropriate restriction enzyme for each mutation was selected using NEBcutter version 2.0 (New England Biolabs, http://nc2.neb.com/NEBcutter2/). Three

Table 2
The five nonsynonymous mutations identified in GluCl gene.

GluCl mutation	Nucleotide mutation	Amino acid change	Localization of mutation	Louse population (ivermectin-HL or Lab-BL)	Pattern of substitutions
Mut-1	T136C	S46P	N-terminal extracellular domain	Ivermectin-HL	Transition
Mut-2	A427G	N143D	N-terminal extracellular domain	Ivermectin-HL / Lab-BL	Transition
Mut-3	A706G	T236A	M3 transmembrane domain	Ivermectin-HL	Transition
Mut-4	C752T	A251V	M3 transmembrane domain	Ivermectin-HL	Transition
Mut-5	A815G	H272R	M3–M4 linker	Ivermectin-HL	Transition

BL, body lice; HL, head lice.

set pairs of primers for amplifying the fragments encompassing the three mutations were designed using Primer3 software, version 4.0 (http://frodo.wi.mit.edu/primer3/). The details of primers, PCR conditions, and restriction enzyme are given in Table 1. The PCR-amplified fragments corresponding to S46P, H272R, and A251V polymorphisms were digested with the restriction enzymes AgsI (cut wild type), NsiI (cut wild type), and HhaI (cut mutated type), respectively. Amplicons were subjected to restriction analysis according to the supplier's instructions. Digested PCR products were separated on 2% agarose gels and the resulting DNA bands were visualized under ultraviolet light.

In total, 76 head lice with suspected ivermectin resistance were collected from Senegal, 40 head lice from the first ivermectin trial and 36 (including the 6 with suspected resistance genotyped above) from the second trial, were genotyped using these PCR-RFLP assays. Sixteen body lice from the laboratory colony, and 24 head lice collected in France and Algeria (stored in our laboratory) were also used as possibly susceptible specimens.

2.4. Statistical analysis

The statistically significant difference of the frequency of each mutation between the samples was calculated using a Chi-squared test using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA; www.graphpad.com) and *P*-values of $\leq$0.05 were considered significant.

2.5. Ethical approval

The study protocol was approved by the National Ethics Committee of the Ministry of Health and Social Action in Senegal (no. 0267MSAS/DPRS/CNRERS). Written informed consent was obtained from the individuals involved or from their legal representatives in the case of children.

3. Results

Firstly, DNA polymorphism analysis from six confirmed clinical ivermectin-resistant head lice, recovered from two Senegalese rural girls, and the laboratory susceptible body lice was performed on the GluCl gene. Large-scale cloning and sequencing (96 clones from 2 independent DNA batches) followed by multiple nucleotides and deduced amino acid sequences comparison revealed the presence of five nonsynonymous mutations: serine to proline (S46P), asparagine to aspartic acid (N143D), threonine to alanine (T236A), alanine to valine (A251V), and histidine to arginine (H272R) (numbering based EEB17068; Fig. 1; Supplementary Fig. S1) resulting from SNPs of thymine to cytosine (T136C), adenine to guanine (A427G), adenine to guanine (A706G), cytosine to thymine (C752T), and adenine to guanine (A815G), respectively, in coding region of GluCl (numbering based XM00242976; Fig. 1).

The N143D and S46P mutations were located at the N-terminal extracellular domain of the channel which carries the glutamate binding site. The T236A and A251V mutations were found in the TM3 transmembrane domain and the H272R mutation in the M3–M4 linker transmembrane region of GluCl. The fact that the N143D mutation occurred in all tested lice, suggests that this mutation is a natural one and has nothing to do with resistance (Table 2). The T236A mutation was found to be located within the binding site of ivermectin, but the fact that it is a conservative amino acid substitution coupled with its low frequency (found only in 3/96 clones) suggests that this mutation is not likely associated with resistance (Fig 1). Therefore, only the three remaining mutations (S46P, A251V, and H272R), found from the head lice with clinical ivermectin resistance, were thought to be potentially associated with ivermectin resistance.

Secondly, to investigate the occurrence of these three mutations (S46P, A251V, and H272R) in all louse specimens collected during the 2 ivermectin clinical trials from Senegal (76 lice) and in order to support their association with ivermectin resistance, 3 RFLP-PCR assays for genotyping were developed. First, these RFLP-PCR assays were approved by testing the known genotype clones. The results showed that the genotypes of three mutations can be detected accurately by these assays (Fig. 2).

RFLP-PCR assays were then used to genotype louse individuals from the studied louse specimens. Louse specimens never exposed to ivermectin were used as susceptible controls (40 lice). As shown in Table 3, none of the three mutations were found in the laboratory susceptible colony (8 body lice tested) or in the other louse specimens used as susceptible controls (32 lice tested), while all the three mutations were detected in clinical ivermectin-resistant head lice collected in Senegal during the two ivermectin clinical trials (76 head lice analysed). The most prevalent mutation was A251V with allelic frequency of 0.33 followed by S46P (0.28) and H272R (0.18) mutations. Considering the years of sampling, the allelic frequency of A251V mutation was significantly elevated from 2014 to 2015, the opposite scenario was observed for H272R mutation, while for S46P the allelic frequency remained stable over the 2 years.

4. Discussion and conclusion

In this study, a field head louse with confirmed clinical resistance to ivermectin [30] was subjected to genetic analysis targeting the GluCl gene, the primary target of ivermectin [24,26,28,29,31]. Through DNA-polymorphism analysis (cloning and sequencing combined with PCR-RFLP genotyping), three relevant non-synonymous mutations in the GluCl gene were identified only in clinical ivermectin-resistant head lice. The A251V mutation found in the TM3 transmembrane domain was the most prevalent, followed by the S46P mutation located at the N-terminal extracellular domain. The H272R mutation detected in the M3–M4 linker transmembrane region of GluCl was the less frequent. Overall, all mutation frequencies were low, possibly suggesting that a selection could be acting on this gene as a consequence of ongoing resistance.

Several studies on GluCl channels have identified different point mutations affecting ivermectin sensitivity in arthropods [24]. In *Drosophila melanogaster,* the P299S mutation located in the M2–M3 linker region of GluCl, has been associated with ivermectin resistance [28]. The A309V mutation in the TM3 transmembrane

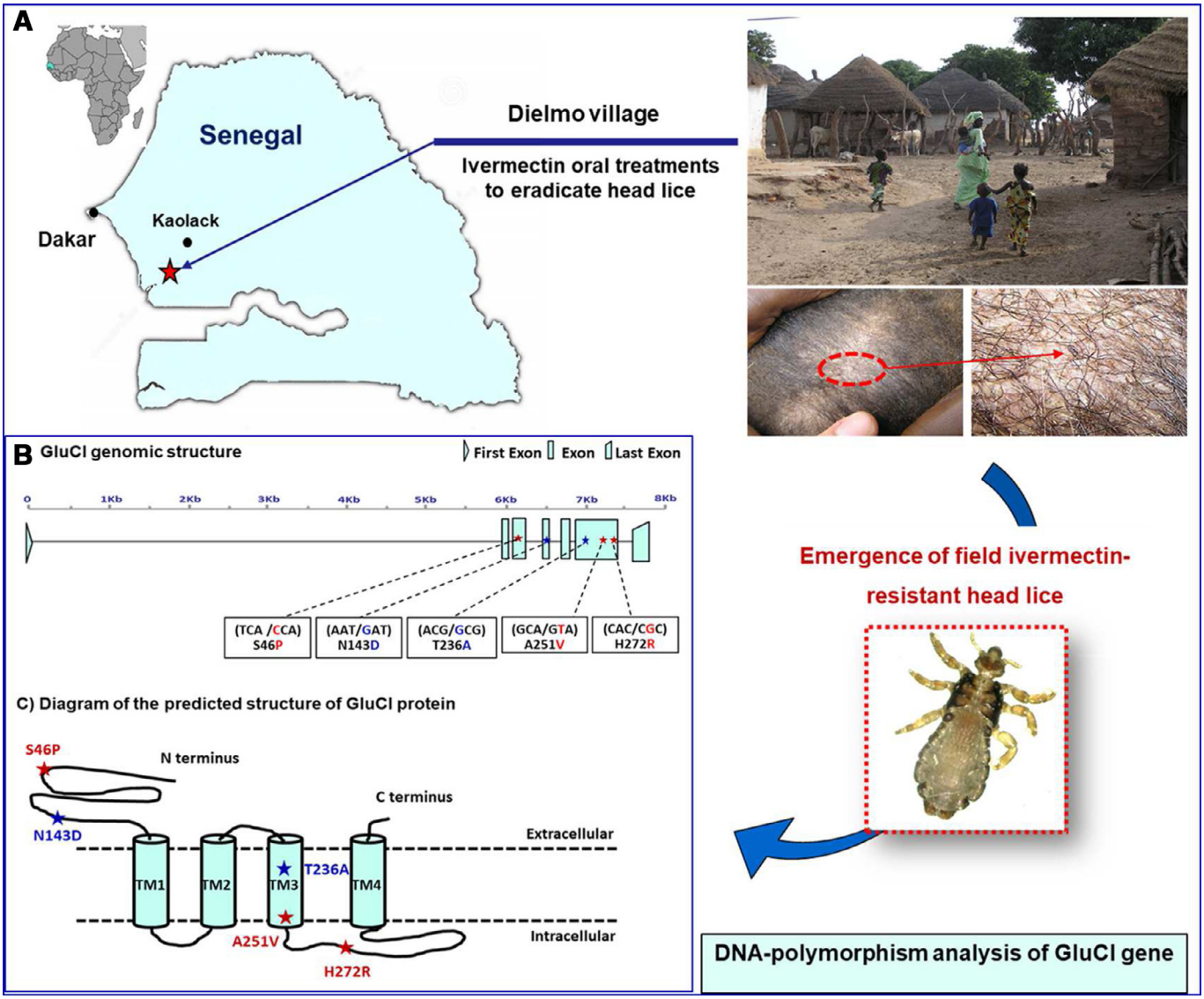

Fig 1. Mutations in GluCl associated with field ivermectin-resistant head lice in rural human populations of Senegal. (A) Map of collection site of ivermectin-resistant head lice in rural human populations of Dielmo village located in the Sine-Saloum area of Senegal. (B) Genetic structure of the GluCl gene, and (C) schematic diagram of the predicted structure of the GluCl protein showing the position of five mutations. Positions of mutations are indicated by stars. Amino acid replacements that may result in structural alterations and found only in head lice with clinical ivermectin resistance are depicted in red. Amino acid positions are numbered according to body lice GluCl protein (accession no. EEB17068).

domain of *Plutella xylostella* GluCl, has been associated with a 10-fold reduction in abamectin sensitivity [27]. In spotted spider mite, *Tetranychus urticae*, the TuGluCl1 G323D and TuGluCl3 G326E mutations found in the TM3 transmembrane domain, were associated with 18- and 2000-fold resistance to abamectin, respectively [24,26].

The three mutations reported herein do not map onto any of the previously reported mutations in other arthropods. Furthermore, whether these mutations contribute directly to ligand binding, or whether they cause conformational changes that influence ligand binding, is currently not known, and requires further characterization. The most prevalent mutation A251V is located at the TM3 transmembrane domain. This transmembrane domain is important in the chloride channel pore formation [22,23]. More importantly, this TM3 transmembrane domain is near to the TM3-Gly36′ residue (corresponding to the Gly232 in *P. humanus* GluCl) identified by site-directed mutagenesis in the avr-14b subunit of *Haemonchus contortus*. This residue is critical as its mutation drastically affects ivermectin potency [32]. The mutation in this residue was also associated with elevated levels of abamectin resistance in wild isolates of *T. urticae* [24,26]. Even if this residue occurs at the ivermectin-binding site, the mutation in this site was found to reduce ivermectin sensitivity by altering the functional properties of the GluCl rather than specifically affect the binding of ivermectin [33]. A sSimilar mechanism can be suggested for the A251V mutation. The replacement of alanine by valine is likely to have significant biophysical and thus structural consequences for the *P. humanus* GluCl with implications for the efficacy of ivermectin binding.

The mutation S46P is located in the glutamate binding site at the N-terminal extracellular domain of GluCl. This location is not in or near reported putative ivermectin binding sites. However, we cannot exclude the possibility that it may be involved in the resistance because N-terminus region can be a structural requirement for ivermectin-induced activation of the GluCl [29,31]. Moreover, in the *Cooperia onchophora* nematode, three (E114G, V235A, and L256F) and two (V60A and R101H) point mutations were found at the N-terminal extracellular domain of GluCla3 and GluClb,

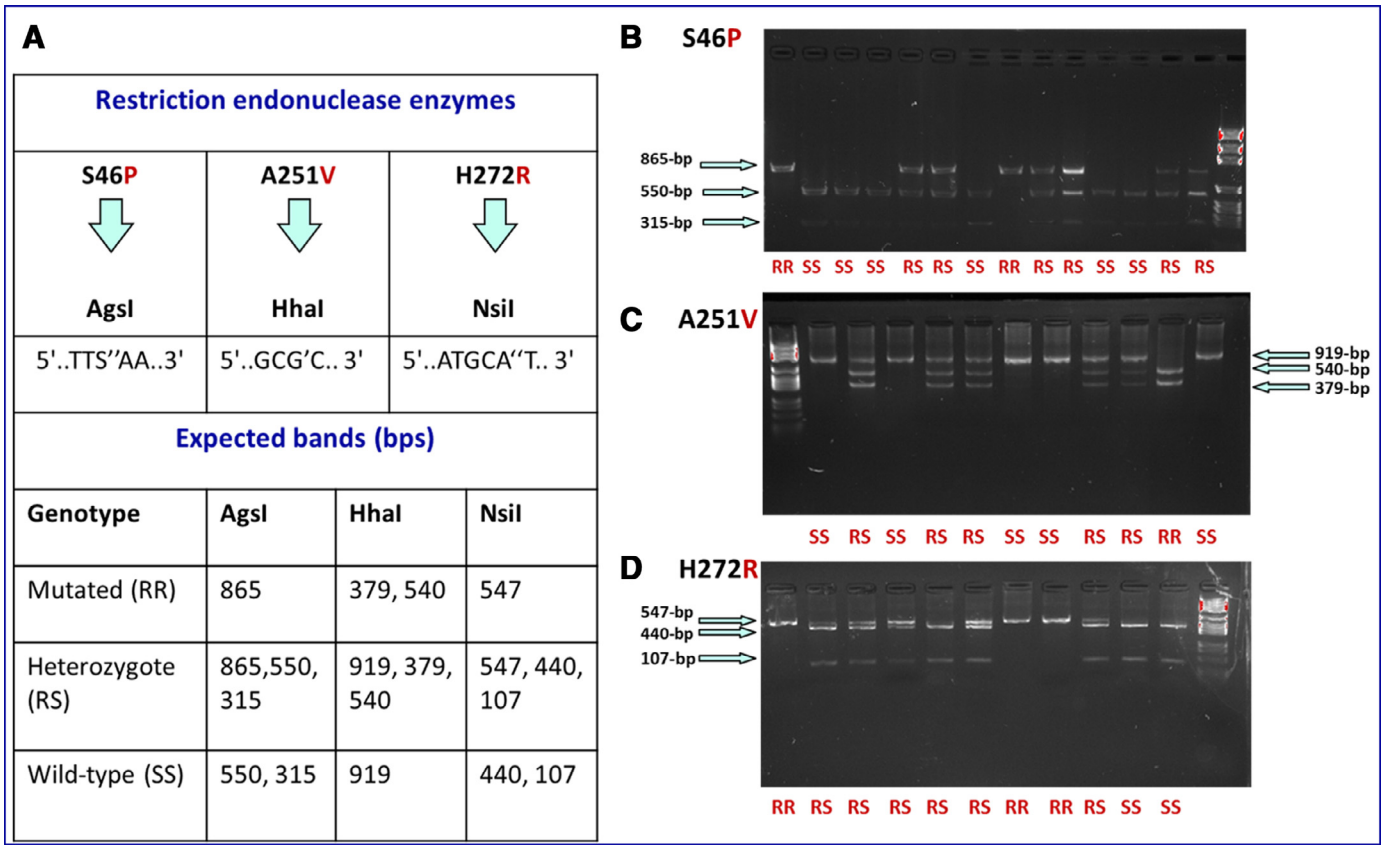

Fig 2. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assays for genotyping S46P, H272R, and A251V mutations of the GluCl gene. (A, upper) The AgsI, NsiI, and HhaI recognition sites are shown above the DNA sequences. (A, lower) The size of the expected fragments for all possible genotypes. (B–D) SYBR safe-stained 2% agarose gels showing the results of digestion with AgsI, NsiI, and HhaI enzymes.

Table 3
Frequencies of S46P, A251V, and H272R mutations using the RFLP-PCR assays in louse specimens.

Lice tested (no.)	Genotype profile	Genotype frequencies: no. (%)		
		S46P	A251V	H272R
HL-Senegal* (ivermectin-trial 2015) (36)	RR	3 (8.3%)	6 (16.6%)	0 (0%)
	RS	16 (44.4%)	20 (55.5%)	6 (16.7%)
	SS	17 (47.2%)	10 (27.8%)	30 (83.3%)
Mutated allelic frequency		*0.30* [- a]	*0.44*[§↑b]	*0.08*[§↓c]
HL-Senegal* (ivermectin-trial 2014) (40)	RR	0 (0%)	3 (7.5%)	2 (5%)
	RS	21 (52.5%)	12 (30%)	17 (42.5%)
	SS	19 (47.5%)	25 (62.5%)	21 (52.5%)
Mutated allelic frequency		*0.26* [a]	*0.22* [b]	*0.26* [c]
	RR	3 (3.9%)	9 (11.8%)	2 (2.6%)
Total* (76)	RS	37 (48.7%)	32 (42.1%)	23 (30.3%)
	SS	36 (47.4%)	35 (46.1%)	51 (67.1%)
Mutated allelic frequency		*0.28*	*0.33*	*0.18*
HL-Algeria (16)	RR	0 (0%)	0 (0%)	0 (0%)
	RS	0 (0%)	0 (0%)	0 (0%)
	SS	16 (100%)	16 (100%)	16 (100%)
Mutated allelic frequency		0	0	0
HL-France (8)	RR	0 (0%)	0 (0%)	0 (0%)
	RS	0 (0%)	0 (0%)	0 (0%)
	SS	8 (100%)	8 (100%)	8 (100%)
Mutated allelic frequency		0	0	0
BL-SDF (8)	RR	0 (0%)	0 (0%)	0 (0%)
	RS	0 (0%)	0 (0%)	0 (0%)
	SS	8 (100%)	8 (100%)	8 (100%)
Mutated allelic frequency		0	0	0
BL-Laboratory colony (8)	RR	0 (0%)	0 (0%)	0 (0%)
	RS	0 (0%)	0 (0%)	0 (0%)
	SS	8 (100%)	8 (100%)	8 (100%)
Allelic frequency		0	0	0

BL, body lice; HL, head lice; RR, resistant homozygote in which both alleles are mutated; RS, heterozygote in which only one of the two alleles is mutated; SS, susceptible homozygote in which neither allele is mutated. A Chi-squared test comparison of the allele frequencies was carried out between HL-Senegal collected in 2014 and 2015. Significant levels: [§↑b] $P<0.05$ compared with [b], [§↓c] $P<0.05$ compared with [c].

* Lice specimens with suspected ivermectin resistance.

respectively [29]. Amongst these mutations, electrophysiological studies determined that the L256F mutation of GluCla3 causes a three-fold reduction in ivermectin sensitivity [29]. The glutamate binding site is also site that harbor mutations in wild avermectin-resistant strains of nematode *Caenorhabditis elegans* [31].

In summary, our study has for the first time associated the possible involvement of three-point mutations on the GluCl gene in clinical ivermectin-resistant head lice occurring in rural human populations of Senegal. No similar mutations have been reported in other studies to date. Therefore, functional expression and analysis of GluCl variants containing these mutations would provide crucial information on the toxicodynamic role of these mutations in determining ivermectin sensitivity.

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Declarations

None.

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Competing Interests

The authors declare that they have no conflicts of interest.

Ethical Approval

This study was approved by the National Ethics Committee of the Ministry of Health and Social Action, Senegal (no. 0267MSAS/DPRS/CNRERS). Written informed consent was obtained from the involved individuals.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi: 10.1016/j.ijantimicag.2018.07.005.

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