

MAJOR ARTICLE

Quinine Treatment Selects the *pfnhe-1* ms4760–1 Polymorphism in Malian Patients with *Falciparum* Malaria

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Background. The mechanism of *Plasmodium falciparum* resistance to quinine is not known. In vitro quantitative trait loci mapping suggests involvement of a predicted *P. falciparum* sodium–hydrogen exchanger (*pfnhe-1*) on chromosome 13.

Methods. We conducted prospective quinine efficacy studies in 2 villages, Kollé and Faladié, Mali. Cases of clinical malaria requiring intravenous therapy were treated with standard doses of quinine and followed for 28 days. Treatment outcomes were classified using modified World Health Organization protocols. Molecular markers of parasite polymorphisms were used to distinguish recrudescence parasites from new infections. The prevalence of *pfnhe-1* ms4760–1 among parasites before versus after quinine treatment was determined by direct sequencing.

Results. Overall, 163 patients were enrolled and successfully followed. Without molecular correction, the mean adequate clinical and parasitological response (ACPR) was 50.3% (n = 163). After polymerase chain reaction correction to account for new infections, the corrected ACPR was 100%. The prevalence of ms4760–1 increased significantly, from 26.2% (n = 107) before quinine treatment to 46.3% (n = 54) after therapy (P = .01). In a control sulfadoxine–pyrimethamine study, the prevalence of ms4760–1 was similar before and after treatment.

Conclusions. This study supports a role for *pfnhe-1* in decreased susceptibility of *P. falciparum* to quinine in the field.

Keywords. Malaria; recurrence; antimalarials; *Plasmodium falciparum*; drug resistance; sodium–hydrogen antiporter; microsatellite; Mali.

Control of uncomplicated cases of malaria has been greatly impaired by development of resistance against mainstay antimalarial agents [1, 2]. The first–line antimalarial strategy is presently artemisinin combination

therapy. Quinine (QN) is used in many malaria-endemic countries for the management of severe life-threatening malaria and as rescue therapy for parasites resistant to other antimalarial drugs. As a monotherapy, QN is associated with significant recrudescence rates [3]. This may be because QN is a rapidly acting drug with a relatively short elimination half–life [4, 5]. Failure of QN in malaria treatment has been long documented, particularly in South America [6, 7] and Southeast Asia [8]. Although high–level resistance of *Plasmodium falciparum* to QN is rare, in vivo and in vitro decreases in parasite susceptibility have led to the use of this drug in combination, particularly with the antibiotics tetracycline or doxycycline [9–13].

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The mechanisms of decreased *P. falciparum* susceptibility to QN are not clearly understood. Modulation of in vitro QN sensitivity has been associated with single nucleotide polymorphisms in the *pfcr* (chloroquine-resistance transporter) and *pfmdr1* (multidrug resistance 1) genes [14, 15]. Involvement of additional genes also seems to be important, because the association of *pfcr* and *pfmdr1* is not always observed [16]. This reality of a complex phenotype is also supported by the frequent efficacy of QN against chloroquine-resistant parasites [17, 18].

The availability of a laboratory-derived genetic cross between 2 parasites of markedly different in vitro QN responses [19] permitted a quantitative trait loci-based approach that identified genomic regions contributing to the phenotypes [18]. Analysis showed a possible association between reduced *P. falciparum* susceptibility to QN and a locus on chromosome 13. A pairwise effect was also detected between that chromosome 13 locus and an additional locus on chromosome 9. The set of predicted genes on the chromosome 13 locus included a homologue to the family of Na⁺/H⁺ exchanger (NHE) proteins [18]. The *P. falciparum*-encoded PfNHE-1 is 1920 amino acids long and harbors an open reading frame with a microsatellite polymorphism consisting of variable DNNND repeat units, designated ms4760. The predicted *pfnhe-1* sequence was analyzed in the Dd2 × HB3 progeny, as well as in a range of field isolates and laboratory strains with variable susceptibilities to QN. Eight variants of ms4760 were originally discovered. The variant ms4760-1 with 2 copies of DNNND repeat units was significantly associated with reduced in vitro QN sensitivity [18]. These results were later supported by transfection experiments [20]. The aforementioned studies were performed with in vitro cultured laboratory strains. However, these polymorphisms were also present in natural *P. falciparum* populations [21] and found to be associated with QN sensitivity among field isolates in in vitro assays [22–24].

Here we extend these studies to clinical settings by exploring the in vivo impact of QN therapy on the prevalence of the *pfnhe-1* ms4760 and *pfcr* polymorphisms in 2 Malian villages.

METHODS

Study Design and Procedures

This QN efficacy study was conducted in Kollé and Faladié, Mali. Kollé (with approximately 2800 inhabitants) is a rural village located 57 km south of Bamako, the capital city of Mali. Faladié (with approximately 3000 inhabitants) is located 80 km northwest of Bamako. Malaria is endemic in both villages, with seasonal transmission occurring between July and December.

Patients presenting with clinical malaria requiring intravenous treatment were eligible for the study. The 28-day World Health Organization (WHO) 2003 protocol for antimalarial

drug efficacy was adapted to the QN regimen [25]. Patients aged ≥6 months who presented with *P. falciparum* malaria at a parasitemia of ≥500 000 trophozoites/μL or who had ≥1 clinical symptom of severe malaria (eg, coma [Blantyre coma scale score of <2], prostration, inability to drink or eat, vomiting, convulsion, obsessing, respiratory distress, icterus, and hematuria associated with the presence of parasites) were included. Informed consent or assent was obtained before any protocol-specific procedure was performed. Pregnant women, patients allergic to QN, and patients whom the physician believed to be too severely ill were excluded from the study. At inclusion, thick and thin blood smears and full clinical examinations were performed for each.

Patients enrolled during 2005–2007 were treated with 12.5 mg/kg QN twice daily for 5 days. In 2008, the regimen was changed to 8 mg/kg 3 times daily for 7 days, according to established protocols (WHO malaria treatment guideline 2006 [WHO/HTM/MAL/2006.1108]). All patients received full curative doses of QN, which was administered by the study team in an inpatient setting. QN treatment was administered intravenously with 10% hypertonic glucose solution until the patient was able to receive oral treatment.

Follow-up was adapted to the QN treatment schedule. Briefly, active follow-up was performed on days 1–7, 14, 21, and 28. Body weight, height, axillary temperature, splenomegaly prevalence, pulse rate, breathing rate, and other signs of a severe condition were measured and recorded on case report forms. For laboratory assessments at enrollment and at each follow-up visit (days 0, 1, 3, 7, 14, 21, and 28 and during unscheduled visits), thick and thin blood films and blotting of blood onto filter paper samples were performed. Thick and thin blood films were stained with Giemsa stain, and parasite density was determined by dividing the number of asexual parasites by 300 leukocytes and multiplying by 7500 (the estimated mean of leukocytes/microliter of blood) [26].

Treatment outcomes were defined according to the clinical or parasitologic response and were classified as early therapeutic failure (ETF), late clinical failure (LCF), late parasitologic failure (LPF), and adequate clinical and parasitologic response (ACPR), according to slight modifications of WHO protocols [25].

Cases of QN treatment failure were treated with artesunate-amodiaquine or artemether-lumefantrine, according to the Malian National Malaria Control Program guidelines. Severe malaria cases that could not be managed in the field clinic were referred to teaching hospitals in Bamako.

As a control, a prospective sulfadoxine-pyrimethamine efficacy study was conducted as described elsewhere in Kollé during 2007 and 2008 [27]. The prevalence of ms4760-1 in baseline and post-sulfadoxine-pyrimethamine infections was measured.

The study was approved by the Ethics Committee of the Faculty of Medicine, Pharmacy, and Odonto-Stomatology,

University of Bamako, Mali, and the Ethics Committee of the National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda.

Molecular Methods

P. falciparum genomic DNA was extracted from blood blotted onto filter paper samples by an established methanol-based method [28].

Molecular markers of parasite polymorphisms *pfmsp1*, *pfmsp2*, and *CA1* were used to distinguish recrudescence parasites from new infections by nested polymerase chain reaction (PCR), as described elsewhere [29, 30]. A sample was classified as a true failure if it was recrudescence with all 3 markers.

The diversity of ms4760 was determined by direct PCR amplicon sequencing in all samples before QN treatment and in all recurrent parasitemia during the 28-day follow-up. Nested PCR was performed for the first PCR amplification, with the following primers and conditions: F1fw 5'-GACAATTTCCGGTAGATGCCCTAT-3' and R1rev 5'-GGTCTTGCACTGCATGGA-3', using 1 × buffer, 1.5 mM of MgCl₂, 200 μM of each dNTP, 1 μM of each primer, and 0.625 U of the Taq DNA polymerase (Invitrogen, San Diego, CA, USA). The product was used to perform the second amplification with the following primers, under the same conditions as the first amplification: F2fw 5'-AGTGGTAGTAATAATGATA-3' and R5rev 5'-GTACCCTTAGTTGAAATATT-3'. Products of approximately 350 base pairs from the second amplification were treated with ExoSAP-IT enzyme, as recommended by the manufacturer (USB, Cleveland, OH). The obtained product was capillary sequenced using the second amplification primers F2fw and R5rev and fluorescent nucleotides in an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA). The prevalence of ms4760-1 among parasites before versus after QN treatment was determined. Conversely, as a negative control, we measured the prevalence of ms4760-1 at baseline and after sulfadoxine-pyrimethamine treatment. DNA was extracted from blood blotted filter papers that was collected before treatment and for any parasitemia after administration of sulfadoxine-pyrimethamine. Sequencing was performed as described above. The polymorphism of *pfhne-1* ms4760 was determined by alignment. The prevalence of *pfhne-1* ms4760 was compared between the pretreatment and posttreatment infections.

Samples collected before and after QN treatment and for which the results from *pfhne-1* analyses were available were tested for the *pfprt* K76T codon, using previously described methods [31].

Quinine Plasma Levels

Plasma QN levels were determined in blood samples collected at 3 time points: before QN administration on day 0, 7 days after the initiation of treatment, and 14 days after the

initiation of treatment. Only patients with samples from the 3 time points described above were included in this pharmacokinetic analysis. Fifty microliters of plasma were used for QN quantification. Quinine was measured by liquid chromatography with spectrometric detection after extraction by a dichloromethane isopropanol mixture in the presence of hydroquinidine chlorhydrate as an internal standard. Detection was by excitation at 350 nm and fluorometric emission at 440 nm, using a flow rate of 2 mL/min, with the injection rate set at 30 μL. A 4.6 mm × 25 cm ultrasphere column with octadecylsilane particles (Beckman) was used (the protocol was developed at Laboratoire de Pharmacologie Analytique et Pharmacocinétique, Hôpital Saint-Vincent de Paul, Paris France).

Data Analysis

Patients were included in the analysis only if they received the correct dose of the study drug, attended all follow-up visits, and did not take another antimalarial drug during the follow-up period.

EpiInfo version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA), SPSS 11.0 (The Predictive Analytics; IBM, Chicago, IL), and STATA 9.1 (StataCorp, College Station, TX) were used to generate summary statistics and to compare therapeutic efficacy. The Spearman correlation test was used to assess the relation between ms4760-1 and the *pfprt* K76T codon mutation. The prevalence of polymorphisms before versus after QN therapy was compared with the Fisher exact test and 2 × 2 contingency table analysis. Sequencher vs 4.7 (Gene Codes, Ann Arbor, MI) was used to align and compare field sample sequences with the previously described 8 variants of *pfhne-1* ms4760 [18].

RESULTS

In Vivo Quinine Efficacy

Overall, 169 patients were enrolled (83 from Faladié and 86 from Kollé). Six were lost to follow-up, yielding 163 patients for the final in vivo analyses. Without molecular correction, the mean ACPR was 50.3% (Table 1). After PCR correction to

Table 1. In Vivo Quinine Efficacy Among Subjects From Faladié and Kollé, Before and After Molecular Correction

Parameter	Faladié, Subjects, % (n = 82)	Kollé, Subjects, % (n = 81)	Overall, Subjects, % (n = 163)
LCF	12.4	23.5	17.9
LPF	39.5	23.5	31.5
ACPR	48.2	53.1	50.3
ACPRc	100	100	100

Abbreviations: ACPR, adequate clinical and parasitological response; ACPRc, adequate clinical and parasitological response after molecular correction; LCF, late clinical failure; LPF, late parasitological failure.



Figure 1. Alignment of ms4760 microsatellite sequences from different *Plasmodium falciparum* lines. *A*, The 8 sequences described by Ferdig et al [18]. *B*, The 10 new sequences from the Malian Quinine study. DNNND copies are highlighted in **bold**. Asterisks indicate base changes to the encoding nucleotide sequence. Insertions are underlined. New sequences found in Mali are classified from ms4760-9 to ms4760-18.

account for new infections, the corrected ACPR was 100% (Table 1). Results for the 2 villages were similar. Overall, 81 patients (49.7%) had a patent infection during follow-up. After the 5-day and 7-day QN treatment regimens, 69 of 126 patients (54.8%) and 12 of 37 patients (32.4%), respectively, presented with posttreatment parasitemia ($P = .01$). The 7-day treatment regimen delayed reinfections by 5 days, from day 14 to day 19 after treatment initiation.

Most (94%) of the 163 patients cleared parasitemia by day 3 after initiation of QN therapy, while all patients had cleared parasitemia and fever by day 7.

Molecular Analysis

All 163 day 0 samples were available for molecular analysis, while only 73 of 81 posttreatment samples were available. Of the 236 samples amplified by PCR, 22.9% were mixed infections and did not yield usable sequences. PCR and sequencing were successful in 107 of 163 samples (65.6%) obtained before QN therapy and in 54 of 73 (74.0%) obtained after QN therapy. The polymorphism of *pfmhe-1* ms4760 was determined by alignment and compared between pretreatment and posttreatment samples.

Overall, 6 of the 8 *pfmhe-1* microsatellites described by Ferdig et al [18] and 10 other ms4760 variants were found. Alignment of these variants showed new deletions or insertions (Figure 1), of which 5 had been observed in other studies with samples from Africa and India [24, 32–34].

ms4760-1 was present in 26.2% of samples (107) obtained before QN treatment but in 46.3% of samples (54; $P = .01$) obtained after QN treatment. The odds of recurrent parasitemia after QN therapy among patient presenting with ms4760-1 at day 0 were 2.4 times the odds among patients who did not

present with ms4760-1 at day 0 (95% confidence interval, 1.2–4.8). In the control sulfadoxine-pyrimethamine study, the prevalence of ms4760-1 before treatment was not statistically different from the prevalence after treatment (Table 2).

There was a trend toward decreased prevalence of ms4760-1 from day 14 (66%; $n = 9$) to later time points (days 21–28; 42.2%; $n = 45$). Interestingly, at each of these time points, the prevalence of ms4760-1 remained higher than at baseline (data not shown).

The ms4760-3 marker, in contrast, was significantly more prevalent before treatment (36.4%) than after treatment (14.8%; $P = .004$). The remaining microsatellites were randomly distributed before and after the QN treatment (Table 3)

Among the samples from both villages, *pfprt* codon 76 analysis was successful in 74 pre-QN and 28 post-QN treatment samples. Mixed infections with both wild-type and mutant alleles were excluded. The prevalence of the *pfprt* 76T codon was similar in the pretreatment samples, compared with the posttreatment samples (Table 4)

Table 2. Selection of ms4760-1 by Quinine but Not Sulfadoxine-pyrimethamine in *P. falciparum* Parasites From Subjects in Kollo and Faladje, Mali

Treatment	Before Treatment, Samples, % (No.)	After Treatment, Samples, % (No.)	P^a	OR (95% CI)
Quinine	26.2 (107)	46.3 (54)	.01	2.43 (1.2–4.8)
SP	31.8 (44)	14.3 (21)	.23	0.36 (.09–1.4)

Abbreviations: CI, confidence interval; OR, odds ratio.

^a By the χ^2 or Fisher exact tests.

Table 3. ms4760 Diversity Among Parasites in Samples Collected Before (Day 0) and After Quinine Treatment From Subjects in Kollé and Faladié

Variant	Prevalence, %		P
	Day 0 Parasites (n = 107)	Recurrent Parasites (n = 54)	
ms4760-1	26.2	46.3	.01
ms4760-2	0	0	NA
ms4760-3	36.4	14.8	.004
ms4760-4	0	0	NA
ms4760-5	1.9	1.85	.5
ms4760-6	3.7	1.85	.8
ms4760-7	22.5	16.7	.3
ms4760-8	3.7	7.4	.5
ms4760-9	0	1.85	NA
ms4760-10	0	1.85	NA
ms4760-11	1.9	0	NA
ms4760-12	0	1.85	NA
ms4760-13	0	1.85	NA
ms4760-14	0.9	0	NA
ms4760-15	1.9	0	NA
ms4760-16	0	1.85	NA
ms4760-17	0.9	0	NA
ms4760-18	0	1.85	NA

The predominant variants are in bold.
Abbreviation: NA, not applicable.

Pharmacokinetics Analysis

Of 30 patients included in the PK study, a subset of 12 showed interpretable results. Among those 12 patients, the mean plasma level of QN was 25 µg/mL at day 7 (interquartile range, 1.88–3.11) but was below the detection limit of 1 µg/mL at day 14.

DISCUSSION

In this study, QN treatment selected malaria parasites with the *pfmhe-1* microsatellite ms4760-1. The observed selection appears to be QN specific, as the treatment with sulfadoxine-pyrimethamine was not associated with such selection of ms4760-1 in posttreatment parasites.

Our data are in agreement with the study by Ferdig et al [18] that found an association between *pfmhe-1* and decreased susceptibility to QN. They are also consistent with other studies that assessed the role of *pfmhe-1* in *P. falciparum* resistance to QN in culture-adapted parasites [16, 33], culture-adapted *P. falciparum* field isolates, or ex vivo experiments [32]. More-recent in vitro studies also point to the involvement of the *pfmhe-1* gene in modulation of QN response in vitro in field samples [33], and Nkruma et al [20] provide transfection data that support a role for *pfmhe-1* in QN resistance, albeit in a

Table 4. pfcr1 K76T Profile Distributions Among Parasites Harboring ms4760-1 in Samples Collected Before and After Quinine Treatment From Subjects in Kollé and Faladié

Profile	Before Treatment, Samples, % (No.)	After Treatment, Samples, % (No.)	P
<i>pfcr1</i> 76T	69.0 (74)	64.3 (28)	.6
ms4760-1/ <i>pfcr1</i> 76T	55.6 (18)	80.0 (10)	.3

strain-dependent manner. Finally, Ugandan field isolates harboring ms4760-1 showed a modest decrease of in vitro QN sensitivity, but the presence of ms4760-1 did not predict clinical failure during the in vivo study [34]. Our results are, however, at odds with other recent reports suggesting that the *pfmhe-1* ms4760 microsatellite was not associated with QN susceptibility in the Republic of Congo, in Asia [23, 35], and in travelers returning to France from various countries [36]. These discordant findings suggest that the role of *pfmhe-1* in QN resistance may be dependent on the genetic background of the parasites [20] and/or geographic origin [23].

When markers of parasite polymorphisms were used to distinguish true recrudescence infections from reinfections, we found that all recurrent parasites were different from the original day 0 parasites. Yet, we show clinical evidence for significant selection of ms4760-1 on initiation of QN therapy. Furthermore, given the low serum levels of QN on day 14—consistent with its pharmacokinetic characteristics—the question arises of how this selection of ms4760-1 would have occurred in vivo. One hypothesis might be that the observed selection was merely due to normal biological fluctuations during follow-up. However, in these same villages, when sulfadoxine-pyrimethamine was used to treat patients, there was no selection of ms4760-1, indicating that the selection observed in the QN study was indeed due to an effect of QN.

Most of the selective effect was observed in parasites appearing in the bloodstream at day 14. This peak of selection just 2 weeks after initiation of treatment raises the possibility that these parasites might have been released from the liver, which could have occurred 6–10 days earlier [37] and then could have been selected by residual low-level QN pressure. It is also possible that the parasites that appeared at day 14 were indeed present at day 0, although at levels undetectable even by nested PCR. QN might have cleared the highly susceptible parasites, while parasites with decreased susceptibility or, possibly, those that were metabolically quiescent could have recovered in numbers to become detectable at day 14 or beyond. This is supported by our observation that, when QN was administered for a longer period, the rate of recurrent parasitemia decreased and the time to patent parasitemia increased. We note that no QN was detectable by HPLC at day 14. This is consistent with results of mathematical models [38] and

with our previous observations, in which pyrimethamine treatment selected DHFR mutant parasites within weeks, although these parasites were undetectable at day 0 [39], as well as with studies performed in Malawi, where the *pfcr* 76T codon was not detected by conventional nested PCR but was detected in 25% of subjects by a heteroduplex tracking assay [40].

The observed efficacy of QN (after molecular correction to distinguish new infections) is consistent with several other reports reviewed by Okombo et al [24]. However, overall, 49.7% of 163 patients had a patent infection during the 28-day follow-up, which is comparable to the failure rate observed in other studies, using a similar treatment dose of 30 mg/day in 3 doses for 5 days [4, 41]. The rate of reinfection on day 14 justified the change of treatment duration from 5 to 7 days, which not only decreased the rate of recurrent parasitemia but also increased the time to patent parasitemia by 5 days. Thus, as previously shown, treatment duration is important in QN's in vivo efficacy [42].

We found that the uncorrected ACPR after QN treatment was 50.3%. These parasites may represent new infections from novel mosquito bites in these settings of intense malaria transmission. As discussed above, these parasites may also be new blood-stage organisms emerging from the liver. In either case, the fact that half of the patients would be sent home with impending parasitemia is of public health concern. This is consistent with several previous reports and supports the current recommendation of intravenous artesunate for the management of severe malaria [43–45]. In areas where intravenous artesunate is not available, QN treatment should be followed with a full course of an artemisinin combination therapy before discharge from the hospital. Alternatively, a drug that kills liver-stage parasites may be a good adjunct to QN therapy.

As shown in several other recent field studies [17, 22, 24, 32, 34, 46], in addition to 6 of the 8 *pfnc* microsatellites described by Ferdig et al [18], we discovered 10 novel microsatellites randomly distributed before and after the QN treatment. This confirms the high diversity of *P. falciparum* in Malian settings. ms4760-3 was the most represented microsatellite in our samples before QN treatment, followed by ms4760-1 and ms4760-7, whereas ms4760-2 and ms4760-4 were not found in this study. Other types, including the new ones, were all in low frequencies. This observation is similar to the East Africa ms4760 profile [18, 24, 34], while the Asian setting harbors mainly ms4760-7 [18, 35]. This diversity in ms4760 in the 2 villages in Mali was not affected by QN treatment. The significance of the counterselection of ms4760-3 by QN treatment was not known.

A few studies [14, 15, 21] have implicated *pfcr* and/or an interaction between *pfcr* and ms4760-1 in the phenotype of decreased susceptibility of *P. falciparum* to QN. We found no selection of *pfcr* by QN treatment in vivo, although a trend for an increase in the ms4760-1 + *pfcr*76T haplotype was

noted. This trend did not reach statistical significance. The role of “ms4760-1 + *pfcr* 76T” in QN resistance in the field needs further investigation in larger studies.

Although QN treatment selected *pfnc*-1 ms4760-1, one might ask whether this microsatellite could be used as a molecular marker for QN resistance. We found that patients who presented with ms4760-1 at day 0 were nearly 2-fold more likely to have a recurring parasitemia after QN treatment, but the confidence interval was too wide for the association to reach statistical significance, probably because of the relatively small sample size. This comparatively small scale of the study was due to the logistic difficulties associated to the performance of these types of in vivo field studies. First, QN is only indicated for severe malaria, which is an unusual event [45]. Second, because of the need for multiple samples, for ethical reasons we could only include patients who were ill enough to warrant QN treatment but not too severely ill to undergo the study procedures. These difficulties are reflected in the fact that it took approximately 4 years to include 169 suitable cases in this study. Finally, to avoid alterations of the parasite population by culture adaptation, we performed direct sequencing of PCR products from blood samples collected directly from the patients, and nearly 23% of the 236 samples indicated mixed infections and did not yield usable sequences. Apart from the obvious difficulties, our study warrants the future conduct of similar but larger studies. Such studies are necessary for confirming the present observation and allow the pooling of data (eg, in the context of the WWARN initiative) for the full assessment of the role suggested here of ms4760-1 as a molecular marker of QN resistance.

In conclusion, our study is the first to demonstrate the selection of *pfnc*-1 ms4760-1 by QN treatment in vivo and supports an important role for this locus in QN resistance in the field.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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