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Population genetics and drug resistance markers: an essential for malaria surveillance in Pakistan

Afsheen Raza

Mohammad Asim Beg
Aga Khan University

REVIEW ARTICLE

Population Genetics and Drug Resistance Markers: An Essential for Malaria Surveillance in Pakistan

Afsheen Raza and Mohammad Asim Beg

ABSTRACT

Plasmodium (P.) vivax is the prevalent malarial species accounting for 70% of malaria cases in Pakistan. However, baseline epidemiological data on *P. vivax* population structure and drug resistance are lacking from Pakistan. For population structure studies, molecular genetic markers, *circumsporozoite protein (csp)* and *merozoite surface protein-1 (msp-1)* are considered useful as these play an important role in *P. vivax* survival under immune and environmental pressure. Furthermore, these genes have also been identified as suitable candidates for vaccine development. While efforts for effective vaccine are underway, anti-malarial agents remain the mainstay for control. Evidence of resistance against commonly used anti-malarial agents, particularly Sulphadoxine-Pyrimethamine (SP) is threatening to make this form of control defunct. Therefore, studies on drug resistance are necessary so that anti-malarial treatment strategies can be structured and implemented accordingly by the Malaria Control Program, Pakistan. This review aims to provide information on genetic markers of *P. vivax* population structure and drug resistance and comment on their usefulness in molecular surveillance and control.

Key Words: *Plasmodium vivax. Genetic diversity. Drug resistance markers. Malaria. Circumsporozoite protein (csp). Merozoite surface protein-1 (msp-1).*

INTRODUCTION

Malaria is a mosquito-borne infectious disease of humans and other animals caused by genus *Plasmodium (P.)*. Five *Plasmodium* species are known to infect humans; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*. Of the five species, *P. falciparum* is the most virulent while *P. vivax* is the most common causing an estimated 216 million annual infections worldwide. Approximately 2.6 billion people are at risk globally of acquiring malaria via this species.[1] Mortality rates worldwide are not documented as yet.

Although, approximately 80 – 90% of *P. vivax* burden is concentrated in the Central and South America, Middle East and Asia,[2] however, baseline data on epidemiology, genotypes, drug resistance and pathogenesis is lacking from key malaria endemic areas including Pakistan.

Epidemiology: Malaria poses a major public health problem for Pakistan with an estimated 4.5 million suspected malaria cases being reported, of which 70% are caused by *P. vivax* and 30% are caused by *P. falciparum*. Pakistan is one of the 6 countries within WHO Eastern Mediterranean Region with areas of high malaria transmission and is thus regarded as a malaria endemic region with 100% of the population living at risk (Figure 1).[3]

Nationally, on the basis of endemicity, the provinces can be divided into three groups: high endemicity province with average five-year Annual Parasite Incidence (API) > 5, moderate endemicity province with average five-year API < 5 and low endemicity province with average five-year API < 1. According to this categorization, all the highly endemic districts belong to Balochistan, Khyber Pukhtunkhwa and some areas of Sindh province. Major reasons for high malaria endemicity in these areas are heavy monsoon rainfalls and floods, poor sanitation, improper management of waste, poor public health care system and high human migration influx into Sindh and Balochistan.[4] These factors provide a safe heaven for the spread of mosquito vectors and facilitate malaria

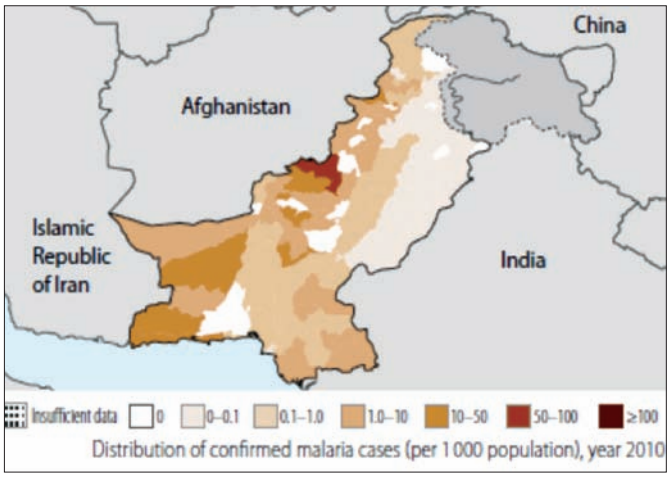

Figure 1: Malaria endemicity in Pakistan (WHO malaria report 2012).

Department of Pathology and Microbiology, The Aga Khan University, Karachi.

Correspondence: Dr. Mohammad Asim Beg, Department of Pathology and Microbiology, The Aga Khan University, Stadium Road, Karachi.
E-mail: masim.beg@aku.edu

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transmission through human parasite reservoirs,[5] possibly increasing malaria burden every year in Pakistan.

Though, a major contributor of malaria, *P. vivax* is a neglected parasite species worldwide and in Pakistan[6] and limited data is available on circulating genotypes, transmissibility and prevalence of drug resistance. Immense epidemiological significance is associated with these aspects and need based research is required so that baseline data could be reported. After such a data is gathered, analyzed and reported to the Malaria Control Program, Pakistan, steps for effective malaria control can be taken and policies implemented in Pakistan.

To perform epidemiological studies on the population characteristics and drug resistance patterns of *P. vivax*, knowledge on the life cycle and the antigens produced by the parasite in the human host needs to be understood.

Life cycle: The life cycle of *P. vivax* includes many transitions and stages in both mosquito and human host. When infected *Anopheles* mosquito bites a human, it releases motile sporozoites into the blood vessels which are transported to the liver. In the liver, the sporozoites enter the hepatocytes, initiate nuclear division and eventually segment resulting in the formation of tens of thousands of merozoites. The clinical disease begins after an incubation of 1 – 4 weeks, when the infected hepatocytes rupture and release the merozoites into the blood stream. The asexual cycle and pathogenic stage begins when the merozoites invade the reticulocytes, develop and multiply further while ingesting the haemoglobin. Some merozoites develop into gametocytes (male and female forms) that await ingestion by the mosquito, thus, initiating another pathogenic cycle in a different human host.[7]

In *P. vivax* infection, frequent relapses are very common (occurring approximately 3 – 6 weeks apart) since the sporozoites sometimes enter a dormant, hypnozoite stage in the liver and become active later on. Factors that trigger the growth of these latent forms are unknown. These relapse patterns of *P. vivax* are highly significant with regard to genetic diversity, drug resistance and disease severity. However, limited data has been published on this aspect of *P. vivax*.[7]

Genetic diversity in *Plasmodium vivax*: Genetic diversity is defined as the total number of heritable characteristics in the genetic makeup of the species.[8] Studies have shown that *Plasmodium* species show diversity in several parameters including morphology, biochemistry, relapse patterns, symptoms, course and duration of infection, immunological responses, drug resistance and transmissibility by anopheline vectors. However, the extent of genetic diversity observed depends on the transmission rates, immune pressure and natural selection of the parasite in an area.[9] Usually in endemic areas where transmission rates are high, extensively diverse variants are circulating posing a threat to the public health since such variants have possibly acquired increased virulence and resistance to drugs in order to fit and survive.[10]

Majority of studies on genetic diversity of *P. vivax* have been based on polymorphic markers encoding parasite surface antigens such as *circumsporozoite protein (csp)* and *merozoite surface protein-1 (msp-1)*.[11] These antigenic genes exhibit nucleotide polymorphisms which result in production of multiple allelic forms.[12] These alleles are capable of successfully surviving within the host and the environment.[13] Thus, knowledge of respective allelic forms within a population helps in understanding the origin, dispersal and stability of various parasite genotypes and their role in drug resistance, host immune response and effectiveness of control strategies.[13] However, due to paucity of data from all provinces of Pakistan, the usefulness of respective markers in malaria surveillance need to be further explored.

***Circumsporozoite protein (csp)*:** *Circumsporozoite protein* is the most abundant polypeptide present on the sporozoite surface. It exhibits extensive polymorphism allowing *P. vivax* to escape the host immune system and thus sustain itself within a region.[11] Therefore, it is the most widely studied candidate antigen for vaccine development against pre-erythrocytic stage of the parasites life cycle.[13]

Structurally, it is a single copy gene comprising a central domain of tandem repeated sequences flanked by two non-repeated conserved sequences (Figure 2). In *Plasmodium vivax*, this central domain is composed of a 27 bp element repeated variable number of times. Two types of non-peptide repeat units; GDRA (A/D) GPQA, namely VK 210 type and ANGA (G/D) (N/D) QPG, namely VK 247 type have been observed commonly in *P. vivax*.[14] A given parasite line can exhibit either VK 210 or VK 247 type.[15] However, mixed genetic infection of both VK 210 and VK 247 is also observed, mostly in areas of high malaria transmission.[6,16,17] Globally, VK 210 is the best adapted variant prevalent in almost all *P. vivax* endemic regions.

Studies on the distribution of *csp* types in neighbouring countries of Pakistan such as India, Iran and

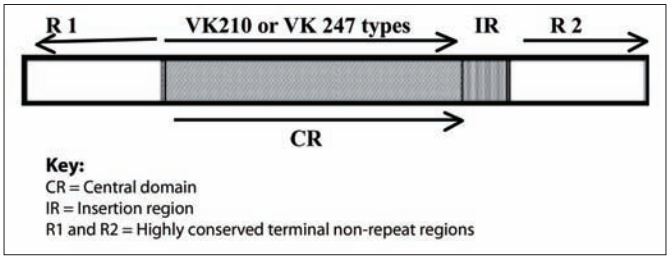

Figure 2: Organization of *Plasmodium vivax* circumsporozoite protein gene.

Afghanistan have shown that genetically distinct *csp* variants are circulating within different areas of the same country for example Kolkata, India reports a prevalence of VK 210 type (99.3%) while Orissa reports a significant prevalence of both VK 210 and VK 247 types (39.5% and 26.3% respectively).[18,19] Similarly, reports from Nangarhar, Afghanistan and Sarbaz/Chabahar, Iran documents a prevalence of VK 210 type (94.8% and 79.2% respectively).[14,19,20] Furthermore, areas such as Orissa, Herat, Sarbaz/Chabahar have also reported significant prevalence of mixed genetic infections of both VK 210 and VK 247 types. Since prevalence of mutant and mixed infection depends on the transmission intensity of parasite in an area as well as the transmissibility of *Anopheline* vector,[14,18] therefore, these studies suggest a high transmission intensity of *P. vivax* in their respective areas. Consequently, such transmission rates put immense host immune pressure on the parasite leading to natural selection of certain genotypes in an area.[9]

Scant data from Pakistan on prevalent *csp* types has been reported. Both studies show variable results on circulating *csp* types. Study on clinical isolates of *P. vivax* from Northern belt of Pakistan (Federally Administered Tribal Areas) reported the predominance of VK 210 type (95.7%) in the respective region.[19] A larger study from southern Pakistan, encompassing Sindh and Balochistan province documented a different pattern with prevalence of VK 247 type in Balochistan (39%) but not in Karachi and rural Sindh where prevalence of VK 210 type (87.4% and 95.2%) was documented.[6] Interestingly, mixed infections were observed in 1.9% and 2.2% of the total samples in FATA and Karachi only.[6,19] These results provide evidence that similar to its neighbouring countries; different provinces of Pakistan also harbor highly diverse *csp* variant types. Therefore, it is recommended that larger studies from all the provinces of Pakistan, especially from high malaria transmission areas be conducted so that true prevalence of *csp* variants could be reported.

The significance of conducting epidemiological studies on *csp* types is that it provides a baseline for assessing the immunological significance of variant types. Previous studies have shown that degree of antibody response, induced in an individual, against *P. vivax* is dependent on *csp* type infecting an individual. Several studies have reported varying response from different endemic areas. Some studies document better antibody response against VK 247 type,[21] while others report that VK 210 type induces more potent immune response than VK 247 type.[22] In Pakistan, prospective serological assays and trials, such as those mentioned above, are only possible when baseline malaria surveillance using *csp* marker is performed on a large scale in different provinces of Pakistan. Eventually, this baseline surveillance data and serological studies can provide guidance in effective malaria vaccine designing.

***Merozoite surface protein-1 (msp-1)*:** *Merozoite surface protein-1* is a 200 kDa protein expressed on *P. vivax* merozoite surface. During maturation, this protein undergoes primary and secondary proteolytic cleavage into four (*msp*-1 83, 30, 38 and 42 kDa) and two fragments (*msp*-1 33 and 19 kDa) respectively. Functionally, during parasite lifecycle, *msp*-1 is a critical antigen involved in red cell invasion. Structurally, *msp*-1 is, as characterized by monkey adapted *P. vivax* cell lines (Belem and Salvadore) show, a mosaic organization of several variable blocks flanked by interspecies conserved blocks (ICBs, Figure 3). ICBs are regions that exhibit dimorphic substitutions and are highly conserved among *Plasmodium* species while the variable blocks exhibit polymorphic substitutions. Numerous recombination sites within conserved and polymorphic regions result in frequent switching of mutations in *msp*-1, leading to generation of new alleles.[12]

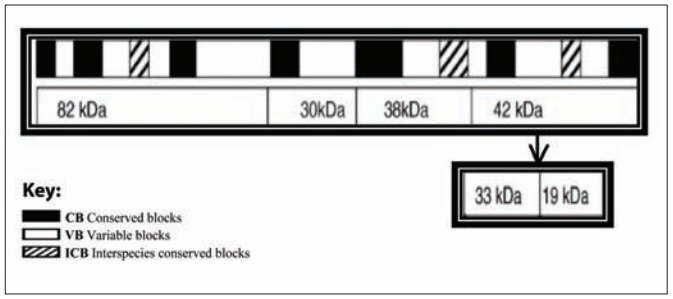

Figure 3: Organization of *Plasmodium vivax* merozoite surface protein 1 gene.

Studies on *msp*-1 allele distribution have been performed from diverse endemic regions and data indicates that three *msp*-1 types, categorized on the basis of structural similarities with Belem and Salvadore-1 strains are circulating globally: type-1 (homologous to Belem), type-2 (homologous to Sal-1) and type-3 (homologous to both Belem+Sal-1). Type-3 variant is a combination of Sal-1 like sequence at the 5' end and Belem like sequence at the 3' end and is hypothesized to have arisen as a result of intragenic recombination between Sal-1 and Belem.[23]

Sequencing analysis of *msp*-1 from different regions of the same country has shown that genetic fluxes are common in this gene. As with *csp*, the prevalence of *msp*-1 types is also found to be inconsistent within neighbouring countries of Pakistan. In Herat and Nangarhar, Afghanistan, type-2 is the most prevalent (45.7% and 63%). However, the percent prevalence of type-1 and type 3 is also high in Herat (26.6% and 26.7% respectively) while type-1 is only 3.7% in Nangarhar.[45] Similarly, in Iran, both Sarbaz and Chabahar have a moderate prevalence of type-1 (26.6%), type-2 (40%) and type-3 (33.4%).[19]

Limited data have been documented from northern and southern belt of Pakistan on the prevalent *msp*-1 types.[6,19] Both studies show variability in prevalence pattern with highly diverse and mixed genetic infections observed frequently. In the Federally Administered Tribal Areas (FATA), type-2 and type-3 were observed in similar prevalence (39.4% and 33.4% respectively) while type-1 is observed in lower frequency (15.4%) as compared to the other two types.[19] In a study from Southern Pakistan encompassing Karachi, Balochistan and rural Sindh, high prevalence of type-2 variants was observed in all the three areas while mixed infections were observed frequently in Karachi,[6] indicating the high transmission intensity of *P. vivax* in both Northern and Southern Pakistan. Though this knowledge is available, however, gaps still exist on distribution of *msp*-1 variants from other provinces of Pakistan.

The importance of *msp*-1 knowledge is similar to that of *csp*. Serological studies on *msp*-1 allelic variants and their role in modulation of immune responses have been performed globally which have documented that *msp*-1 induces potent-B and T-cell responses that result in reduced parasitaemias in animals and primates. Therefore, *msp*-1 has been identified as a strong candidate antigen for vaccine production against *P. vivax*.[24-26] However, variability in allelic variants distribution may serve as a significant hindrance in the deployment and designing of a *msp*-1 formulated vaccine. Therefore, malaria surveillance reporting information on population genetic markers could serve useful in vaccine designing.

Antimalarial drug resistance in *Plasmodium vivax*: Data on genetic diversity serves as a foundation for drug resistance associated studies as it gives insight about the pattern of dispersal and stability of multilocus genotypes. Such information is essential for predicting and monitoring the usefulness of combining specific drugs, dispersion of and emergence of drug resistant parasites.[13]

Treatment of *P. vivax* infection is recommended with chloroquine (CQ).[5] However, physicians treating patients in the public sector are aware of CQ resistance in *P. falciparum* and inadvertently correlate the same pattern for *P. vivax*. Therefore, the most economical choice of antimalarial prescribed against *P. vivax* in Pakistan is Sulphadoxine-Pyrimethamine (SP).

SP resistance in *P. vivax* has long been documented from worldwide and in northern belt of Pakistan.[27-34] The possible factors documented for SP resistance are untrained laboratory staff that make errors in microscopically diagnosing *P. vivax*, especially in case of mixed infections. In such cases, patients are treated with sub-therapeutic doses of SP,[33] unrestricted use of antifolates, especially Sulphadoxine, for the treatment of diarrhea, urinary tract and respiratory tract infections in Pakistan, that may have resulted in development of intrinsic sulphadoxine resistance in *P. vivax*, single and inappropriate combination antimalarial therapy, prescribed to all who present with fever and chills.[34] All these factors increase the susceptibility of *P. vivax* to accumulate SP resistance associated mutations in its genes in order to survive the environmental drug pressure thus escalating drug resistance situation.

It is possible that due to the above mentioned factors, extensive SP resistance has developed in other areas of Pakistan as well. Thus, monitoring of SP resistance is important since a large number of treatment failure cases serve as a human reservoirs of resistant strains. Further transmission of these resistant strains via mosquito vectors allows the stable transmission of resistant genes in an endemic population,[26] thus, weakening implementation of control strategies by malaria control program.

Genetic markers and mechanism of Sulphadoxine-pyrimethamine resistance in *Plasmodium vivax*: Genetic markers involved in SP resistance in *P. vivax* include *dihydropteroate synthase (dhps)* and *dihydrofolate reductase (dhfr)*. Both these genes produce key enzymes for *de novo* tetrahydrofolate (THF) synthesis and DNA replication. Briefly, *de novo* synthesis of tetrahydrofolate in the parasite begins when folate is reduced to dihydrofolate by the help of *dhps* enzyme. Dihydrofolate is then reduced to tetrahydrofolate followed by synthesis of methionine, glycine and dTTP by *dhfr* enzyme. Parasite DNA synthesis ensues, resulting in active malarial infection.[35]

Sulphadoxine-Pyrimethamine acts as antimalarial drug by inhibiting *dhps* and *dhfr* leading to decreased levels of fully reduced tetrahydrofolate, conversion of glycine, methionine synthesis, thymidylate levels and subsequent arrest of DNA replication (Figure 4).[36] However,

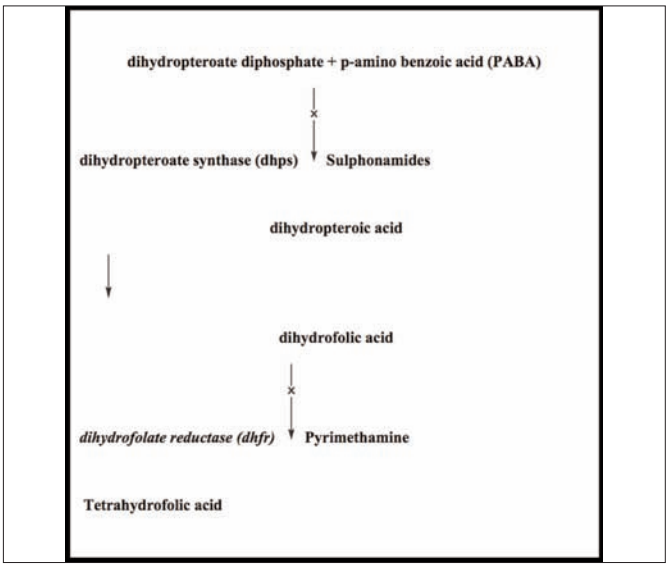

Figure 4: Flowchart showing action of Sulphadoxine-Pyrimethamine on dhfr and dhps enzymes for termination of parasite DNA synthesis.

P. vivax develop resistance against SP via accumulation of Single Nucleotide Polymorphisms (SNPs) in genes coding for *dhps* and *dhfr*.[37] SNP is a DNA sequence variation (mutation) that changes single nucleotides in the genome consequently leading to change in the amino acid sequence (codon) and resulting in production of a different protein. A conformational change in the active binding site of enzymes occurs, thus, affecting drug binding.[38] In *P. vivax*, these SNPs result in decreased SP accommodation, binding and entry thus, making SP ineffective.[39] DNA synthesis continues and a pool of drug resistant strains is maintained in a region.

Studies have shown that SP is useful only against wild type or single mutated alleles. Presence of double, triple, quadruple mutations (accumulation of multiple SNPs) in major binding sites for SP i.e. codon positions 382, 383, 512, 553, 585 in *dhps* and 33, 50, 57, 58, 61, 117, 173 in *dhfr*,[28,29,40] is indicative of emergence of drug resistance making SP defunct for further use against *P. vivax*. Other novel SNPs at different codon positions in *P. vivax* have also been reported. However, their role in SP drug resistance is yet to be established.

Prevalence of SP resistance has been documented extensively from the neighbouring countries of Pakistan. Afghanistan and Iran have reported prevalence of wild type and double mixed mutant alleles of *dhfr* while limited mutations of *dhps* have been documented in both the regions.[16,40] India, on the other hand, reports extensive mutations (upto quadruple) in both *dhfr* and *dhps* implying that SP pressure is high in India compared to its neighbours.[27,28,30] From Pakistan, two studies, one from FATA region and other from Bannu district,[32,33] have been performed contributing to the data base. Both studies report limited mutations in both *dhfr* and *dhps* gene. However, limitations with respect to sampling area (Afghan refugee areas) were inherent in these studies as the issue of cross border migration and its role in prevalence of drug resistance needs to be taken into consideration.

Therefore, the main concern for researchers, at this time, is to gather and analyze data, from different regions of Pakistan, so that Malaria Control Program, Pakistan can map the extent and existence of SP resistance. Formulation of rational policies is dependent on these baseline studies, so that the spread of drug resistance can be controlled.

DISCUSSION

Baseline epidemiological studies on *P. vivax* population structure and drug resistance patterns are the mainstay of research and contribute greatly in understanding as well as controlling *P. vivax* infection effectively. It also allows researchers to estimate the transmission intensity of the parasite within an area so that rational control measures can be undertaken.

In Pakistan, limited data is available on circulating genotypes and drug resistance. Therefore, it is suggested that large scale studies be undertaken so that prevalent genotypes of *csp* and *msp*-1 can be identified. The significance of detecting prevalent genotypes is in the development of effective vaccine. *csp* and *msp*-1 based vaccine formulations has been tested extensively in malaria endemic regions of Central and South America.[22,26,41-43] Interesting results with potent immune responses and parasitaemia clearance have been observed in animal studies. However, these studies have been conducted on sera obtained from individuals residing in the respective areas and the results observed cannot be extrapolated for Pakistani population. The immune responses in every population are distinct depending on both host, environment and parasite factors and, therefore, it is possible that the vaccines developed may not be effective against *P. vivax* variants circulating in Pakistan. Therefore, large scale studies, primarily on *P. vivax* genotypes and secondarily on serological studies is recommended. Once the immune response patterns against *P. vivax* are understood, animal studies and eventual field trials can be performed. The entire spectrum of *P. vivax* research is thus based on the foundation of circulating genotypes.

The knowledge on drug resistance patterns serve as a basis of malaria control. Since, no vaccines are currently available; the main source of control is via anti-malarial drugs. However, with reports of extensive accumulation of resistance associated mutations in *P. vivax*, this form of control seems to be reaching a point of redundancy. Newer and better formulations with combination drugs are recommended for malaria control. However, task of priority from malaria endemic regions is extensive reporting of drug resistance data on *P. vivax*. This will help in mapping and monitoring the extent of drug resistance that may possibly have developed in *P. vivax*. Once this baseline data is available, WHO and the National Malaria Control Programs will be able to develop and implement rational drug policies with respect to the regions.

CONCLUSION

Lack of genetic diversity and drug resistance data are hindering the control of *Plasmodium vivax* transmission in Pakistan. Evidence based research is needed which will enable understanding of population structure and prevalence of resistance so that effective control strategies may be developed and implemented.

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