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Prevalence of Dihydrofolate Reductase and Dihydropteroate Synthase Polymorphisms in *Plasmodium falciparum* isolates from Wad Madani, Gezira state, Central Sudan

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Abstract: Background: *Malaria drug resistance to Sulfadoxine-pyrimethamine (fansidar) resistance had increased all over the world threatens millions of exists. Polymerase Chain Reaction-restriction fragment length polymorphism (PCR-RFLP) methods were used as a tool for monitoring* ***Plasmodium falciparum*** *insider resistance strain in Wad Madani central Sudan.* ***Materials and Method:*** *This study was carried in Marengan clinical center at Wad Medani. One hundred, and seventy-six (176) febrile patients were screened. Forty-four patients (25%) were studied to identify Dihydropteroate synthetase gene (DHPS) 540 and Dihydrofolate reductase gene (DHFR) 108, 59, 51 polymorphisms by DNA spotted on filter paper (day 0) analysis for aforementioned polymorphism by (PCR-RFLP) methods.* ***Results:*** *The results of dhfr gene codons 108, 59, and 51 (n= 44) showed that 14/44 (31.8%) were dhfr 108 mutant and 24/44 (54.5%) were wild types, 3/44 (6.8%) were found as a mixed infection. One sample 1/44 (2.2 %) was dhfr 59 mutant and 31/44 (70.4. %) were wild type, while* ***in*** *codon 51 only 2/44 (4.5%) were dhfr 51 mutant and 28/44 (63.6%) were wild type, 3/44 (6.8%) were found as a mixed infection. Screening of dhps 540 gene we found 2/44 (4.5%) were mixed infection and 42/44 (95.5%) were wild type.* ***Conclusions:*** *In conclusion, the mutations in the dhfr and dhps genes are less abundant, but most likely increasing and thus fansidar resistance may rapidly increase. However, the current studies still indicate the effectiveness of sulfadoxine-pyrimethamine (S/P) as chemotherapy, in comparison to the chloroquine which has been confirmed extremely resistance*

Keywords: *Malaria, Drug-resistant, DHFR, DHPS; Sudan.*

Introduction:

Malaria drug resistance was demarcated as the talent of a parasite strain to multiply or survive; regardless of the mode of action and the dosages of a given drug when compared with dosages that usually recommended, but it should be within the target limits of tolerance that being able for the subjects to bear. [3, 4]

Sulfadoxine-pyrimethamine (S/P) is one of the cheapest alternative and a few effective drugs that have had

Some advantages and relatively a few side effects, good tolerance, and excellent compliance (single oral dose therapy). However, his potential downside is concentrated on the prompt emergency of parasite resistance. Moreover, this drug is massively employed and validated in South East Asia, where the combination of the drug is no longer being In the late 1980s, Sulfadoxine –pyrimethamine sensitivity is ongoing declining in Africa. Confrontation is a prompt acquisition ground on this continent. Therefore, resistance is being less in the west than in the east. However, in east Africa, the degree of resistance is being much mutable. In endemic regions of Tanzania, 1994, a higher percentage of RII/RIII responses was found to be well recognized in children. [10]

Malaria is a main infectious health hazard in the tropics, with 300-500 million annually conquered new clinical cases; most of which are defined as an uncomplicated type of malaria. An outrageous number estimated at the rate of 1.1-2.7 million victims occur each year as a result of a severe invasion of falciparum malaria.[1] The mortality rate arises in sub-Saharan Africa where malaria is an endemic parasitic ailment of Plasmodium falciparum (P. falciparum) infection.[2]

very effective. [5, 6] Sulfadoxine- pyrimethamine resistance was first renowned on the Thia- Compodia borders in the mid of 1960s. [7] Currently, the highest level of resistance was initiated in, Southern China, the Amazon basin, and a large part of South Asia. [8, 9]

In vitro study that piloted in Kenya throughout (the 1980s - 1990s) bared that a low sensitivity of both Sulfadoxine and pyrimethamine together with the presence of pyrimethamine resistance isolates in 1980 and even before Sulfadoxine – pyrimethamine was extensively used [11].The outcomes of the study were successfully documented across sub-Saharan Africa. This study indicates the fluctuation in frequency rate ranges from 13to30% transversely Tanzania during

1994s.[12] Equatorial Guinea in 1990-92s [13] ,and Gana 1991s. [14].

***Falciparum* anti folate resistance genes**

The antifolate Sulfadoxine and pyrimethamine is the core drugs used for the treatment of chloroquine-resistant *P. falciparum* in Africa. Resistance to pyrimethamine has been associated with pointed mutations in the Dihydrofolate reductase gene *(dhfr-gene)*, and resistance to Sulfadoxine with mutations mainly concentrated in the Dihydropteroate synthetase gene (*dhps*-gene).[15]

Dihydrofolate reductase (dhfr) gene

Pyrimethamine acts by selectively inhibiting Dihydrofolate reductase (dhfr) in the malaria parasites and *P. falciparum* resistance, *in vivo* and *in vitro*, have been associated with specific pointed nice mutations in the *dhfr* gene which located on chromosome 4. [16] Asn 108 essential for pyrimethamine resistance, degree of resistance increases with additional mutation I leu 51, Arg 59, or triple mutations. [16]

Dihydropteroate synthetase (dhps) gene

Sulfa drugs performed by selectively inhibiting Dihydropteroate synthetase (dhps) earlier in the folate pathway of the parasite. The gene encoding dhps has been sequenced in *P. falciparum*, which located on chromosome 8, and pointed mutations have been identified and associated with *In-vitro* sulfadoxine resistance under low or no folate conditions. [16]

To date, more than 25 different combinations of *dhfr* and *dhps* alleles have been observed and registered for field isolates parasite. A previous study conducted in Malawi exhibited that the infected patients with parasites carrying the dhps Gly- 437, Glu- 540 double mutant and the dhfr triple Asn 108/I leu 51-Arg 59 mutant have had a specifically high relative risk of treatment failure than those who infected with parasites carrying the dhfr triple mutant alone – such a quintuple mutant (3dhfr and 2dhps mutation) was suggested as a relevant molecular marker for the failure of S/P treatment of uncomplicated *P .falciparum* cases in Blantyre, Malawi. [16]

Material and Methods:

Study subjects

One hundred and seventy six patients with symptoms or signs of suggesting malaria infection were screened for the presence of malaria parasites in their peripheral blood using microscopy. The majority (94%) of infections were *P. falciparum* while the remainders were mixed infection *P. falciparum* and *P. vivax* or *P. vivax* only. Forty four patients which represent 25% were further studied to identify DHPS 540 and DHFR 108, 59 and 51 polymorphisms.

Molecular analysis

DNA Isolation from filter spots using methanol method

DNA prepared from filter paper blood samples, using methanol extraction method as described by Djimde [17]

Polymerase Chain Reaction (PCR)

DNA amplification

DNA extracted from *P.falciparum* collected blood samples and they prepared to be subjected to PCR amplification.[18] DHFR and DHPS genes were examined using primers flanking polymorphic regions of the genes. Two rounds were carried out using outer and inner primers of the two genes. [19]

PCR Protocol for outer PCR reaction

PCR was performed in 30 µl volumes which contains 1X PCR Buffer II (Gene Amp® 10X Buffer II [100 mM Tris-HCl pH 8.3, 500 mM KCl], (Cinna Gen Inc., Iran) 1.5 mM $MgCL_2$ ($MgCl_2$, Applied Biosystem), 0.2 mM each of the dNTP (Gene Amp® dNTPs, Applied Biosystem), 1.0 µ mol of each sense and antisense primers (Table 1), 1 U of AmpliTaq Gold (Applied Biosystem), 5 µl of the Template DNA; and the reaction volume was completed to 30 µl by ddH_2O.

PCR Protocol for Nested PCR reaction

Nested PCR, 2 µl from the outer PCR product was used as a template for the inner PCR. Forty µl volumes which contains primers specific for DHFR & DHPS genes were used.

Following PCR, about 5 µl of each PCR were mixed on parafilm paper with the loading buffer (0.25% bromophenol blue, 25% ficoll, 10mM Tres and 1mM EDTA) and then placed into the gel lanes. The products were electrophoretically separated on 2% agarose gel in Tris-Borate-EDTA (TBE) (0.09M boric acid, 0.09 M Tris, 0.002M EDTA) buffer containing ethidium bromide (5mg / ml) to visualize the DNAs. DNA molecular weight markers (Boehringer Mannheim, U.K), or a 100 bp ladder, were run in parallel wells. The gel was run for about 10-20 minutes in 1x TBE at 120 volts. The gels were examined using GDS (Gell DocMega).

Screening of Resistant genes polymorphisms by Restriction Fragment Length Polymorphism (PCR - RFLP)

When a mutation creates or abolishes a restriction side for specific enzyme, the cleavage with that enzyme can be used as verification for the presence or absence of that mutation. Digestion products which were visualized by electrophoresis result was classified into wild type, pure mutant and mixed.

RFLP analysis of DHFR and DHPS PCR fragments (Duraisingh M.T. et al 1998).

DHFR mutation at position 51

The nested PCR 113 bp was subject to 2 µl NEB U of buffer10x, then 6 µl H2O and 0,2 µl EcoR1 1 restriction enzyme (10 U/ µl), then the mix was incubated over night at 37° C (water path). Samples were run on a 10% poly acrylamide gel for 2 hours. After digestion the original 113 bp fragment was cut in to 78 and 35 bp products.

DHFR mutation at position 59

The nested PCR 113 was subject to 2 µl NEB U buffer10x, 0,2 µl B SA 100 x, 6 µl H2O and 0,2 µl Bsrg 1 enzyme (10 U/ µl), then the mix was incubated over night at 37 C (water path),the samples were ran at a 10% poly acrylamide gel for 2 hour. After digestion the original 113 bps fragment is cut in to 65 and 43 bps products.

DHFR mutation at position 108

25 µl nested PCR product of each sample was digested in the original PCR tube, by adding 3 µl NEB 2 buffer 10x, 0,2 µl Alu 1 enzyme (10 U/ µl) the mix was incubated over night at 37 °C (water path) .The samples were taken on a

10% polyacrylamide gel and run for 2 hours. After digestion, the original 254 bps fragment is cut in to 210 and 46 bps products.

DHPS mutation at position 540

25 µl nested PCR product of each sample was digested in the original PCR tube, 3 µl NEB buffer 10x and 0,2 µl Fok 1 enzyme (10 U/ µl) was added, the mix thus was incubated overnight at 37 C (water path).The samples were taken on a 10% polyacrylamide gel and run for 2 hours. After digestion, the original 201 bp fragment was cut in 145 and 56 bps products.

Electrophoresis on 10% polyacrylamide Non-denaturing gel

Digested PCR products were loaded on 10 % non-denaturing polyacrylamide gel (30% Protogel [Acrylamide/Bis-acrylamide, 37.5:1]) provided by (FMC BioProducts) and electrophoresed at 90 V for two hour. Then the digested fragments were stained in 1µg/ml ethidium bromide solution for 10-15 minutes and visualized with UV light in GDS.After digestion the original 254 bps fragment is cut into 210 and 46 bps products.

Statistical Analysis

Statistical analysis was performed by using appropriate statistical tests and measures, according to Statistical Package for Social Science **(**SPSS).

Results:

This study was conducted to investigate the prevalence of DHFR and DHPS polymorphisms which associated with antimalarial drug resistance of different isolates from Gezira state central Sudan by using PCR-RFLP molecular techniques for recognition of resistance site.

Patients' characteristics

The characteristics of the studied population are shown in (Table: 1). The age of the patients recruited in this study ranging 0.5-50 years, while the sex ratio in the study group is shown in (Fig: 1). A percentage of 54.5% (24/44) were registered in females and 45.5% (20/44) in males. The prevalence of disease and parasiteima is described by Madani.[20]

Result detect by RFLP

The results of polymorphism mutations of SP resistance isolates which detected by restriction fragment length polymorphisms technique is shown in (table: 2). DNA was extracted from 47 samples of *P.falciparum* to study DHFR 51, 59,108 mutations, that confer pyrimethamine resistance. Similarly, DNA was extracted from 47 samples of *P .falciparum to* study DHPS 540 mutations that confer sulfadoxine resistance.

The prevalence of dhfr108 polymorphisms

Forty-three out of forty-seven samples (91.5%) were investigated for the prevalence of the DHFR108 mutation. The results indicate that 17(39.5%) were dhfr 108 mutant and 26(55.3%) were wild type .Figure:1. shows the length difference in base pair of wild, and the mutant type (254 bps the wild type & 210, 46 for the mutant type respectively.)

The prevalence of dhfr 59 polymorphisms

Thirty-five out of forty-seven samples (74.5%) were tested for the prevalence of the DHFR 59 mutation, one sample (2.9 %) was dhfr 59 mutant and 34(97.1) were wild type.(Table:2and 3). Whereas, Fig: 2 show the difference in base pair length of the mutant, and wild type (113 bps of the mutant & 65, 43 for the wild type consecutively.)

The prevalence of dhfr 51 polymorphisms

Forty-one out of forty-seven samples (87.2%) were investigated for the prevalence of the DHFR 51 mutation, 5(12.2%) were dhfr 51 mutant and 36(87.8%) were wild type. The difference in length of base pair in the wild, and mutant type (113 bps for the wild type & 65, 43 for the mutant type is illuminated in (Fig: 2) correspondingly).

The prevalence of dhps 540 polymorphisms

Forty-seven out of forty-seven samples (100%) were tested for the prevalence of gene mutation at position 540 the results indicate that 2 (4.3%) were mutant and 45(95.7%) wild type (Fig: 4.) shows the difference found in length of base pair of the wild, and mutant type (202 bp the wild type & 56,145 for the mutant type consistently).

Table: 1. Show Patients characteristics

Characters	N (%)
Females	25/47(53.2%)
Sex	
Males	22/47(46.8%)
<Age (years)	
< 5	5/47(10.6%)
5-9	10/47(21.3%)
10-14	10/47(21.3%)
>14	22/47(46.8%)
Residence	
Urban	40/47(85.1%)
Semi-urban	6/47 (12.8%)
Rural	1/47(2.1%)

Table: 2. Displays the polymorphisms *in dhfr* and *dhps* genes for (47) isolates of *P.falciparum*

Sample No.	DHFR (mutation at position 51)	DHFR (mutation at position 59)	DHFR (mutation at position 108)	DHPS (mutation at position 540)
Q1	W	W	M	W
C31	W	W	M	W
C48	W	M	M	W
C19	W	W	W	W
A36	W	ND	M	M
C33	W	W	W	W
C20	ND	ND	ND	W
A28	M	W	W	W
A30	ND	ND	W	W
C28	W	W	M	M
C16	W	ND	M	W
C40	W	W	M	W
C35	W	W	ND	W
A38	ND	ND	W	W
C49	W	ND	W	W
C43	W	W	W	W
A35	W	W	W	W
C51	ND	ND	W	W
A31	W	W	M	W
A37	W	W	M	W
A15	W	W	W	W
C46	W	W	W	W
C13	W	W	W	W
C5r	W	ND	W	W
A29	M	W	M	W
C47	W	W	W	W
C50	W	W	W	W
A20	M	W	W	W
C38	W	W	W	W
Q2	W	W	W	W
A27	W	W	W	W
A32a	W	W	W	W
C42a	W	W	M	W
A21	W	W	ND	W
A26	W	W	W	W
C29	W	ND	M	W
C27	W	W	W	W
C25	W	ND	W	W
C41a	W	W	W	W
C41b	ND	ND	M	W
C24	W	W	W	W
C23	M	W	ND	W
C37	ND	ND	M	W
C32	W	W	W	W
C34	W	W	M	W
C42b	W	W	M	W
A32b	M	W	M	W

1 2 3 4 5 6 7 8 9 10

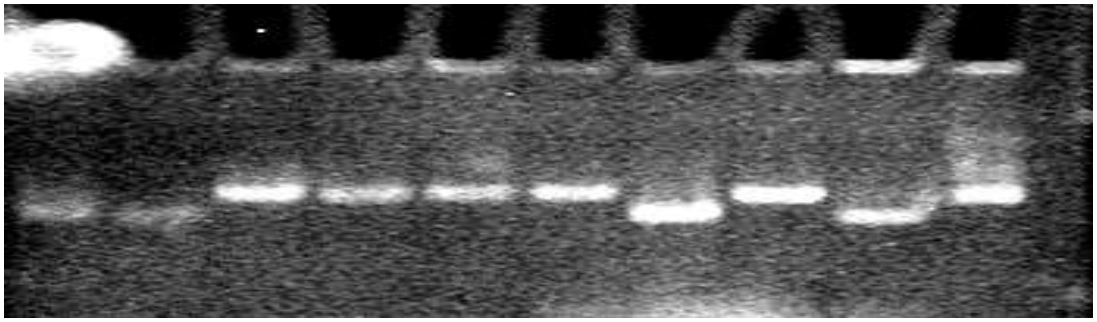

Fig: 1. Displays the PCR-RFLP screening of *P. falciparum* isolates for DHFR codon 108 polymorphisms. Lane 11: DNA markers. Lane 9: digested PCR product (positive control). Lane 1, 2&7: 108 polymorphisms (cut by Alue1 restriction enzyme to210 and 46 bp). Lane 3- 8 &10 are 108 polymorphisms (uncut full length 254 bp).

1 2 3 4 5 6 7 8 9 10

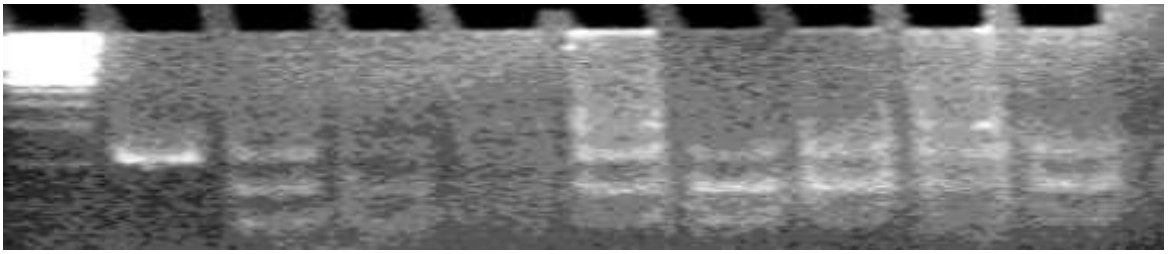

Fig: 2. Clarifies the PCR-RFLP screening of *P. falciparum* isolates for DHFR codon59 polymorphism Lane 1 DNA marker, Lane 2: undigested PCR product (113bp). Lane 3, 4, &6-10 are: 59 polymorphisms (cut by Bsrg 1 restriction enzyme to 65 and 43 bp) sensitive.

1 2 3 4 5 6 7 8 9 10

Fig 3: Illustrates the PCR-RFLP screening of *P. falciparum* isolates for codon DHFR 51 polymorphisms.
Lane 1 DNA marker, Lanes 2-6&8-10 are (uncut full length 113 bp). PCR product (sensitive), Lane 7:51 polymorphisms (cut by EcoR1 restriction enzyme to78 and 35 bp).

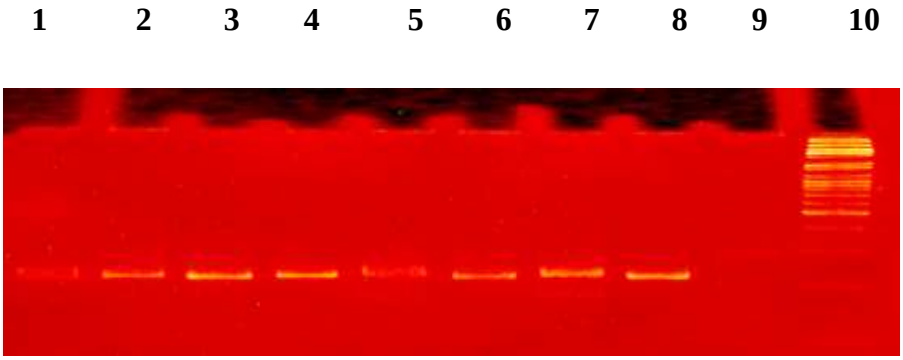

Fig 4: Illuminates the PCR-RFLP **screening of *P. falciparum* isolates for DHPS codon 540 polymorphism**
Lane10: DNA marker. Lane 9: digested PCR product (positive control), Lane 7&5:540 polymorphisms (cut by Fok1 restriction enzyme to 145 and 56 bp) and Lane 8, 6 & 1-4: 540 polymorphism (uncut full length 202 bp).

Table: 3. summarizes the molecular markers results obtained by the current study:

N	Wild types	Mutant types	Mixed types	ND
DHFR108 (47)	26 (55.3%)	14 (29.8%)	3 (6.4%)	4 (8.5%)
DHFR 59 (47)	34 (72.3%)	1 (2.1%)	0	12 (25.6%)
DHFR51 (47)	36 (76.6%)	2 (4.2%)	3 (6.4%)	6 (12.8%)
DHPS540 (47)	45 (95.7)	2 (4.3%)	0	0

Note: ND (not determined)

Discussion

In the Gezira area Malaria is a communal health problem; the predominant parasite species is *P. falciparum* (90%), followed by *P.vivax, P.malariae, and P.ovale.* The main vector is *Anopheles arabiensis.* [21] *Plasmodium. Falciparum* resistance to SP is conferred by point mutations in parasite DHFR and DHPS, the enzymes targeted by these drugs. This allows the use of standard PCR techniques for the monitoring of SP efficacy.

In the contemporary study patients attending Marengan Health Center during the malaria transmission season, were included in the study. Forty-seven samples were conducted to investigate the prevalence of DHFR and DHPS polymorphisms associated with antimalarial drug resistance of different isolates from Gezira state central Sudan by using PCR-RFLP molecular techniques.

Previously, no studies at the molecular level in any of the Sudanese states attempt to test the emergence of chloroquine and sdx /pyr as in 1990 a pregnant woman resident in Sinnar was had *plasmodium falciparum* malaria and was tested for both *in vivo* and *in vitro* response to chloroquine and sdx /pyr. Parasitaemia was cleared on day 1 and no sexual stages or gametocytes seen in day7.[22]

The PCR genetic analysis results obtained from the contemporaneous study revealed that the target mutations conferring SP are 9.6% (mutant type). Besides, more than 71% of the cases were characterized as (wild type).while 4.5% of the cases were found as a mix infection represented by the mutant and wild in the same sample. This result displays a remarkable reduction when compared with those

reported (13.3%) by Yousif, [23] He who observed a noteworthy reduction in SP resistance at Gezira state as it compared with other areas in Sudan (he reports less percentages than that reported in, Khartoum in 2002).
The rate of parasites harboring the mutations was found to be less than those detected in Bahr Elgazal - South Sudan in 2003. In Khartoum, Hag Yousif hospital, Pyrimethamine/Sulfadoxine efficacy was assessed the frequency of dhfr and dhps mutations in 37 *Plasmodium falciparum* isolate that being sampled before subjected the patient to the treatment. Point mutations were detected only at codons 51 and 108 of dhfr and codon 436 of dhps. The frequency of dhfr 51/108 and dhps 436 mutations was 79% and 8%. The plasma levels of SDX indicated an adequate drug absorption by all patients. The presence of alle 51 and Asn 108 mutations among parasites that cleared after treatment indicates that these mutations are merely insufficient to cause in vivo resistance. These consequences suggest that the presence of alle 51/108 dhfr mutations Ala 436 dhps confer decreased susceptibility of Plasmodium *falciparum* to PYR/SDX in area of low endemicity.[24]

Interestingly, these outcomes were instituted to be less than those found in east and West Africa continent. Remarkably, the resistance area wherever, the combination of sulfadoxine-pyrimethamine was particularly scoring high rates and being detected in the northeastern of Tanzania, where the clinical failure rates exceedingly recorded up to 50% as have been described by Mutabingwa.[25] However, high levels of resistance have been also recorded in many parts of Burundi, Kenya, Rwanda, and Uganda as stated by Greenwood. [28] Furthermore, Levels of resistance to sulfadoxine-pyrimethamine are generally lower in West Africa, although the clinical failures are not infrequent, and parasites carrying mutations associated with resistance to sulfadoxine-pyrimethamine are distributed broadly across West Africa[26]. Genetic studies show that resistance to sulfadoxine-pyrimethamine may have been arisen in southern and eastern Africa in a few occasions only, so these resistant strains have spread rapidly [27]. S/P seems to have an unpromising future as the first-line antimalarial in Africa. Despite these results that come from Africa and the gametocytaemia follows treatment, SP is still clinically effective it proved by the current study and can bear an effective impact in malaria control programs, especially when it recommended to be used in a combination with other drugs such as Artensunate to prevent the cumulative mutations and to withstand its low in vivo resistance.

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