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Clinical Pearls

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The first case of artemisinin treatment failure of *Plasmodium falciparum* imported to Oman from Tanzania

Amit Kumar Subudhi, PhD[1], Anne-Lise Bienvenu, PhD[2,3], Guillaume Bonnot, MSc[2], Reem Abu-Shamma, BSc[1], Faryal Khamis, MD[4], Hussain Ali Abdulhussain Al Lawati, PhD[5], Stephane Picot, PhD[2,6,†], Eskild Petersen, MD[4,7,8,†], and Arnab Pain, PhD[1,9,†,*]

[1]Pathogen Genomics Group, Bioscience Program, BESE Division, King Abdullah University of Science and Technology (KAUST), Thuwal, Kingdom of Saudi Arabia, [2]Univ Lyon, Malaria Research Unit, UMR 5246 CNRS-INSA-CPE-University Lyon1, Villeurbanne, France, [3]Service Pharmacie, Groupement Hospitalier Nord, Hospices Civils de Lyon, Lyon, France, [4]Department of Infectious Diseases, The Royal Hospital, Muscat, Oman, [5]Department of Haematology, The Royal Hospital, Muscat, Oman, [6]Institute of Parasitology and Medical Mycology, Hôpital de la Croix-Rousse, Hospices Civils de Lyon, Lyon, France, [7]Institute for Clinical Medicine, University of Aarhus, Aarhus, Denmark, [8]Emerging Infections Task Force, European Society for Clinical Microbiology and Infectious Diseases (ESCMID), Basel, Switzerland, and [9]International Institute for Zoonosis Control, Global Institution for Collaborative Research and Education (GI-CoRE), Hokkaido University, Sapporo, Japan

*To whom correspondence should be addressed. Email arnab.pain@kaust.edu.sa, @pathogensKAUST1
†These authors contributed equally to this work.

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Since its first description and rapid spread in South-East Asia,[1] the emergence of artemisinin-resistant (ART-R) parasites in different pockets of Africa has already started increasing[2–4] and this would pose a major public health crisis.

Case presentation

A 32-year-old male Omani citizen (identified here as Pfa-alm) was admitted to the Royal Hospital, Muscat on 3 June 2018 with a peripheral temperature of 39.8°C after being febrile for a week with no malaria prophylaxis before hospital admission. Before the admission, the individual had a travel history to Dar Es Salam from Muscat, where he spent a night before returning. The patient presented with headaches and muscle pains and was not fully oriented. Giemsa-stained blood film at admission confirmed malaria infection with > 100 000 *Plasmodium falciparum* parasites per microliter (referred to as PfA-alm-preD) collected before drug treatment. Two hours after admission, the patient received intravenous artesunate (2.4 mg/kg) at 0 h, 12 h, 24 h, 36 h and 48 h. On 6th of June, 72 h after admission, oral treatment with artemether/lumefantrine (20 mg of artemether and 120 mg lumefantrine) was started with a single dose of four tablets, followed by four tablets after 8 h and then four tablets twice a day for the following 48 h. Rapid clinical improvement was observed, and even though he was not free from parasites, the patient was discharged afebrile after 7 days with instruction to return if becoming febrile. The patient was readmitted 15 days after discharge with fever, headache and general muscle pains and with a temperature of 39.2°C and parasitemia of 1500 per μl (Table S1). The parasite collected after drug treatment is referred to as PfA-alm-postD. No travel outside Oman during this period was reported (Oman is free of malaria). The patient was treated with mefloquine 1000 mg at admission and 500 mg after 24 h. The patient was afebrile after 3 days and there was no sign of relapse during weekly follow-up for 4 weeks. WHO defines the adequate response to the artemisinin and its derivatives as being microscopy negative on Day 3. The patient with Pfa-alm *P. falciparum* infection had a delayed response and did not become parasite-free before discharge (Figure 1A) which suggests that Pfa-alm is an ART-R *P. falciparum* clinical isolate based on criteria set by WHO (WHO 2018). The parasite clearance half-life of Pfa-alm during the log-phase at H0, H24 and H48 was

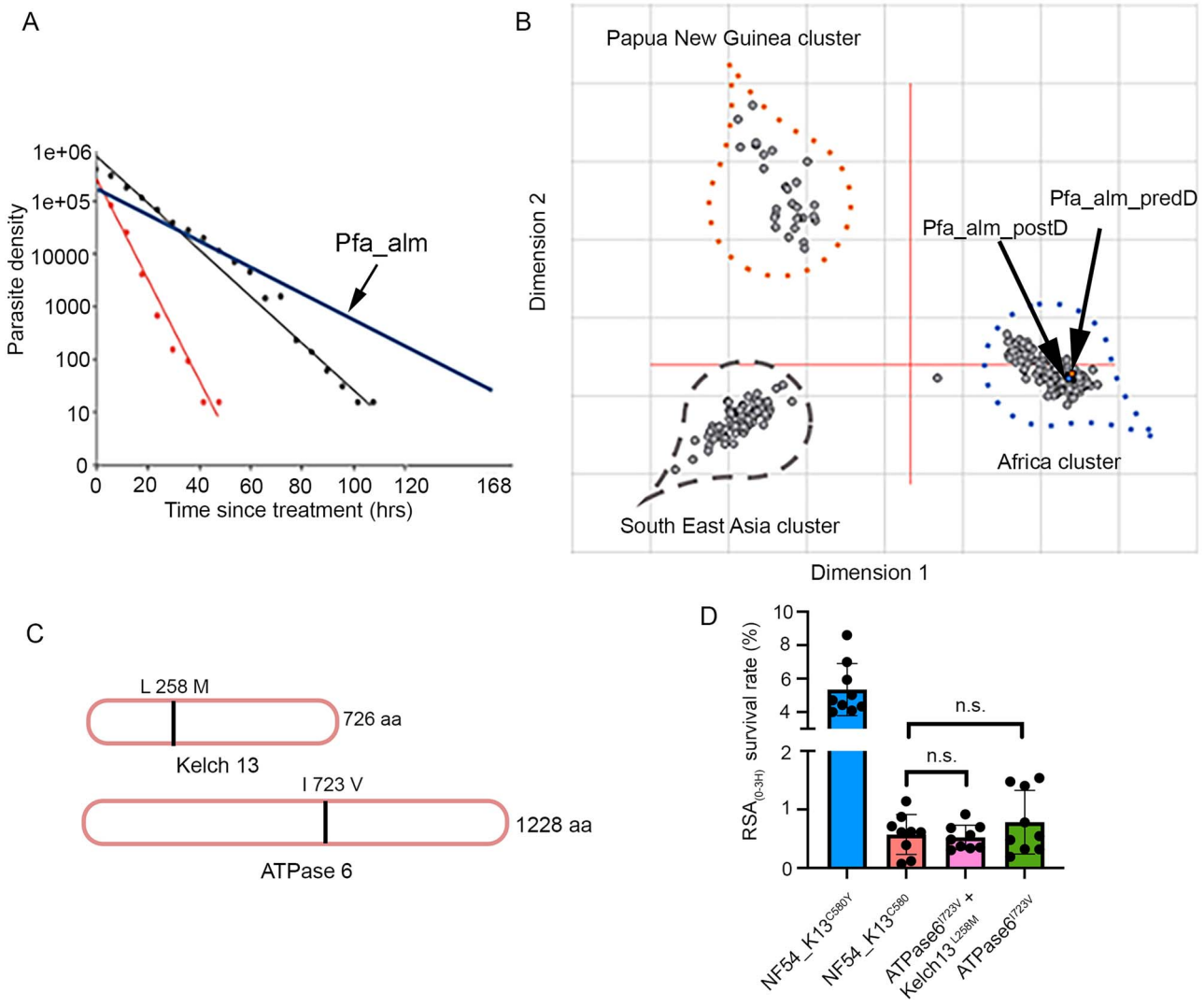

Figure 1. Artemisinin resistance in an imported *P. falciparum* isolate of African origin in Oman. (A) Half-life of the parasite density for Pfa-alm isolate from Oman plotted to graph[5] showing the clearance rates of wild-type *P. falciparum* and genotype with delayed clearance rates. Black and red lines show patterns of parasite clearance from two Thai patients[5] with slow (black) and fast (red) clearance rates after treatment. (B) Principal component analysis of Pfa_alm_preD, Pfa_alm_postD and 247 other *P. falciparum* isolates with different geographic origins. Each dot represents an isolate. The isolates are colour-coded as follows: Pfa_alm_preD, orange; Pfa_alm_postD, blue. Of the 26 918 SNPs, Pfa_alm_preD and Pfa_alm_postD had a total of 53 and 52 SNPs predicted by the Genome Analysis Tool Kit (GATK) program that re-confirmed the geographical origin of Pfa_alm_preD and Pfa_alm_postD parasites. Isolates were clustered into three clusters based on geographic origins (Africa, Papua New Guinea and South East Asia) and demarcated by dotted lines around them. African cluster contains isolates from Kenya, Mali and Burkina Faso, and the South East Asia cluster contains isolates from Thailand and Cambodia. Pfa_alm_preD and Pfa_alm_postD cluster with other isolates from Africa. For a further detailed analysis of the same dataset, please see Figures S1 and S2. (C) NS mutations identified in two candidate genes i.e. *Kelch13* and *ATPase6* for artemisinin resistance. (D) Survival rates of NF54K13 ^{C580Y}, NF54K13 ^{C580}, ATPase6 ^{I723V}, ATPase6 ^{I723V} + K13 ^{L258M} lines in the ring stage survival assay ($RSA_{0\text{-}3h}$). Mean ± s.e.m. $RSA_{0\text{-}3h}$ survival rates were determined from three independent experiments with three technical replicates in each experiment for each parasite line. n.s. not significant (two tailed *T* test). Live parasite's parasitemia followed by survival rate was estimated after counting 2 50 000 un-gated events for each line in each assay

calculated to be 13 h which is significantly delayed compared with the threshold (5 h) which suggests that Pfa-alm has *in vivo* artemisinin resistance.

Genetic analysis of Pfa-alm clinical isolate

We confirmed that Pfa-alm (both PreD and PostD) parasites had originated in east Africa by performing whole genome sequencing and comparing their single nucleotide polymorphism (SNP) profiles with previously sub-selected SNPs of 245 *P. falciparum* isolates collected globally (see Supplementary Materials, Figure 1B, Figures S1 and S2). A comparison of SNPs between Pfa-alm-preD and Pfa-alm-postD confirmed both isolates to be the same parasite (Figure S3), thus ruling out the possibility of any re-infection. Sequencing of the parasite genome revealed one non-synonymous (NS) mutation (mutation that alter the amino acid sequence) in the *Pfkelch13* gene changing the Leucine at position 258 to Methionine and another NS mutation in the SERCA gene (*PfATPase6*) changing the Isoleucine at position 723 to a Valine. These mutations are amongst the NS mutations identified in the known or putative drug-resistance marker genes including those implicated in artemisinin resistance

(Figure 1C and Table S2). The NS mutation in *Pfkelch13* falls outside the propeller domain region. Although many NS mutations in the propeller domain region of PfKelch13 have been validated to confer artemisinin resistance, NS mutations that fall outside of the propeller domain have never been implicated in artemisinin resistance. Based on the previous studies, we suspected these two NS mutations in Pfkelch13 and PfATPase6 proteins to be the most promising and laboratory testable candidates for conferring artemisinin resistance. Both Pfa-alm-preD and Pfa-alm-postD isolates were not cryopreserved for adapting it to culture, hence ruling out the possibility of further study of the artemisinin resistance phenotype of the Pfa-alm isolate in the laboratory. To evaluate if the artemisinin resistance observed in the Pfa-alm parasite was conferred by NS mutations in the *PfATPase6* and *Pfkelch13* alone or in combination, we generated two mutant lines using *P. falciparum* NF54 wild-type laboratory strain (i.e. parasites with no mutation in *Pfkelch13*) using the CRISPR/Cas9 method (see Supplementary Materials, Figure S4 and Table S3). We generated one parasite line with only the I723V variant of the *PfATPase6* and the other with both the I723V variant of the *PfATPase6* and the L258M variant of *Pfkelch13*. We sequenced both the parasite lines to confirm that there were no unintended mutational changes that occurred during the process of introducing the desired mutation. *In vitro*, ring-stage survival assay ($RSA_{0\text{-}3h}$) showed no significant difference between parasites containing only the I723V mutation in the *PfATPase6* or both I723V mutation in the *PfATPase6* and the L258M mutation in *Pfkelch13* and wild-type NF54 parasite line in terms of percentage of survival of parasites in assays analysing at least 2 50 000 cells in each Fluorescence-activated Cell Sorting-based protocol (Figure 1D). In this study, we show that the I723V mutation in the *PfATPase6* and the L258M mutation in *Pfkelch13* do not contribute to artemisinin resistance in the NF54 genetic background.

In conclusion, this study provides evidence about the first reported case of artemisinin tolerant *P. falciparum* strain in a patient imported to Oman that was acquired in Tanzania (East Africa) in 2018. We also conclude that multiple factors such as the genetic background and the role of NS mutations in non-*kelch13* might contribute to reduced susceptibility to artemisinin combination therapy. This report supports calls for major surveillance in Tanzania and other East African nations to monitor for the presence of ART-R parasites circulating in the community.

Supplementary data

Supplementary data are available at *JTM* online.

Author's Contributions

Conceptualization, A.K.S., E.P., S.P., and A.P.; Methodology, A.K.S., R.A.S., and A.P.; Clinical Investigation, E.P., S.P., A.B., G.B., F.K. and H.A.A.A.L.; Analysis, A.K.S.; Writing original draft, A.K.S.; Writing-Review and Editing, A.K.S., E.P., S.P., A.B. and A.P.; Funding acquisition, A.P.; Supervision, A.P. All authors read and approved the manuscript.

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Conflict of interest

The authors have no conflicts of interest to declare.

Data availability and ethics statement

Whole-genome sequences of both the isolates mentioned have been deposited in the European Nucleotide Archive with the study accession number PRJEB44728 and the sequence files are accessible under accession numbers ERR5869704 and ERR5869705. All the research procedures used in this study were approved by the Institutional Biosafety and Bioethics Committee (IBEC) in KAUST (19IBEC12) and the participant involved in this study gave written informed consent via approved procedures at the Royal Hospital, Muscat.

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