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Molecular genotyping in a malaria treatment trial in Uganda – unexpected high rate of new infections within 2 weeks after treatment

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Summary

Polymerase chain reaction (PCR) genotyping of malaria parasites in drug efficacy trials helps differentiate reinfections from recrudescences. A combination therapy trial of one ($n = 115$) or three ($n = 117$) days artesunate (1AS, 3AS 4 mg/kg/day) plus sulphadoxine–pyrimethamine (SP) *vs.* SP alone ($n = 153$) was conducted in Mbarara, a mesoendemic area of western Uganda. All paired recurrent *Plasmodium falciparum* parasitaemias on days 7, 14, 21 and 28 post-treatment were genotyped by PCR amplification and analysis of glutamate-rich protein (*glurp*) and merozoite surface proteins (*msp*) *1* and *2* genes to distinguish recrudescent from new infections. A total of 156 (1AS = 61, 3AS = 35, SP alone = 60) of 199 paired recurrent samples were successfully analysed and were resolved as 79 recrudescences (1AS = 32, 3AS = 8, SP = 39) and 77 as new infections (1AS = 29, 3AS = 27, SP = 21). The ratios of proportions of new to recrudescent infections were 0.2, 0.9, 1.4 and 1.9 on days 7, 14, 21 and 28, respectively ($P < 0.001$, χ^2 test for linear trend). Unexpected high new infection rates were observed early in follow-up on days 7 [5/26 (19.2%)] and 14 [24/51 (47.1%)]. These results impact significantly on resistance monitoring and point to the value of genotyping all recurrent infections in antimalarial trials.

keywords genotyping, recrudescent, re-infections, *Plasmodium Falciparum*, malaria, combination therapy, Uganda

Introduction

The World Health Organization (WHO) *in vivo* antimalarial efficacy testing protocol is instrumental in assessing and monitoring the emergence and extent of parasite resistance to antimalarial drugs (WHO 1973). The current WHO *in vivo* protocol for high transmission areas recommends 28 days of follow-up, stipulating that recurrent parasites should be genotyped by polymerase chain reaction (PCR) to distinguish recrudescent from new infections (WHO 2003, 2006). The ability to discriminate newly acquired infections by comparison of baseline and recurrent parasite genotypes allows a more accurate estimate of the true levels of treatment failures. However, because of resource constraints in malaria-endemic areas, genotyping all recurrent infections, particularly in large trials, is expensive and may not be feasible. To compensate for such limited recourses, correction of treatment outcome could be made by only genotyping post-day 14 recurrences whilst assuming that most recurrent parasites before or on day 14 are likely to be due to recrudescences. This strategy was adopted for a series of WHO/TDR coordinated clinical trials assessing artesunate in combination with standard antimalarial drugs for the treatment of paediatric falciparum malaria in several African countries, including Burkina Faso, Gabon, The Gambia, Sao Tomé, Senegal, Uganda, Malawi and Kenya (von Seidlein *et al.* 2000; Adjuik *et al.* 2002, 2004; Gil *et al.* 2003; Obonyo *et al.* 2003; Priotto *et al.* 2003; Sirima *et al.* 2003). We report the PCR-defined reinfection rates on days 7, 14, 21 and 28

in the Ugandan efficacy trial (Priotto *et al.* 2003) and assess their effect on the efficacy outcome.

Methodology

A 28-day antimalarial combination efficacy trial was conducted in Mbarara District Hospital in Uganda, an area of seasonal, mesoendemic malaria using artesunate (AS 4 mg/kg/day)/placebo plus standard dose sulphadoxine–pyrimethamine (SP) for treating acute uncomplicated, falciparum malaria in children: SP alone ($n = 168$), SP + AS 3 days ($n = 126$), SP + AS 1 day ($n = 126$). The trial profile and clinical findings are detailed in Priotto *et al.* (2003).

PCR amplification

Blood for PCR analysis was collected onto Isocode stix® (Schleicher & Schull, Dassel, Germany) on days 0, 7, 14, 21 and 28 and DNA extracted following the manufacturer's instructions (i.e. washing and boiling of Isocode stix®). *Plasmodium falciparum* in paired samples collected on day 0 and any day (7, 14, 21 or 28) of recurrent parasitaemia were genotyped by analysing the *glurp*, *msp* 1 and 2 loci. PCR amplifications were performed at the Ifakara Health Research and Development Centre (IHRDC) laboratory using an MJ Thermal Controller PTC-100™ (MJ Research Inc., Watertown, USA). Primary *glurp*, *msp1* and *msp2* PCR reactions were multiplexed whereas nested PCR amplifications were performed separately for each locus. The primary and nested amplifications were carried out in 20 and 30 μl reaction volumes using 5 μl of template DNA and 2 μl of primary PCR product, respectively. All oligonucleotide primers and reference DNA were obtained from the Malaria Research and Reference Reagents Resource Centre (MR4). The oligonucleotide primers have been described elsewhere (Felger *et al.* 1994; Irion *et al.* 1998; Snounou *et al.* 1999). Temperature cycling parameters were: initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min (for primary PCR) or 58 °C for 2 min (for nested PCR) and extension at 72 °C for 2 min. The last extension cycle was prolonged for 10 min. *msp2* PCR product (10 μl) was digested with 3 U of *Hin*fIII for 2.5 h at 37 °C and the resulting fragments were resolved on 10% polyacrylamide gels. The *glurp* and *msp1* PCR products were directly resolved on 2% agarose gel. For better comparison of fragments paired samples were loaded onto the gels side by side. Gels were stained with ethidium bromide, visualized under UV illumination, photographed and discrimination made as described.

Definitions of recrudescent and new infections and data analysis

A recurrent parasitaemia was classified as a recrudescence (true failure) if the following conditions were met: (i) all alleles of the three loci in the baseline and recurrent parasitaemia were identical, (ii) a sharing of baseline and recurrent alleles but with some missing alleles in the recurrent parasitaemia, and (iii) a sharing of baseline and recurrent alleles but with new alleles in recurrent sample that were not observed at baseline. A recurrent parasitaemia was classified as a new infection or treatment success, if the allelic pattern for any one of the loci differed completely between the baseline and recurrent samples (Snounou & Beck 1998). The clinical and molecular genotyping data were analysed using STATA v 8.0 (Stata Corporation Inc., Texas, USA)

Results

Of the 373 patients who completed the 28-day follow-up 190 had recurrent parasitaemias. All these recurrent parasitaemias were genotyped by PCR amplification to distinguish recrudescence from reinfection. Table 1 provides a summary of genotyping results per treatment arm. PCR was unresolved for 34 patients due to eight incomplete paired samples and failure to extract/amplify DNA on 26 recurrent samples. The proportions of new infections on days 7, 14, 21, 28 were 5 of 26 (19.2%), 24 of 51 (47.1), 29 of 50 (58%) and 19 of 29 (65.5%), respectively. The corresponding values for the recrudescences were 21 (80.8%), 27 (52.9%), 21 (42%), and 10 (34.5%). Figure 1 illustrates proportions of new and recrudescent infections on days 7, 14, 21 and 28. The ratios of proportions of new to recrudescent infections were 0.2 (19.2:80.8), 0.9 (47.1:52.9), 1.4 (58:42), and 1.9 (65.5:34.5), on days 7, 14, 21, and 28, respectively. The chi-square test for linear trend was 12.8 ($P < 0.001$), demonstrating a linear increase in the odds ratios on days 14, 21 and 28 compared with day 7. The frequencies of new infections increased

Table 1 Distribution of recrudescences, new infections and unresolved recurrent infections in a cohort of Ugandan children treated for *Plasmodium falciparum* malaria

Genotyping status	SP	SP + 1AS	SP + 3AS	Total
Recrudescence	39	32	8	79
New infection	21	29	27	77
Unresolved	14	11	9	34
Total	74	72	44	190

SP, sulphadoxine–pyrimethamine; 1AS, 1 day artesunate; 3AS, 3 days artesunate.

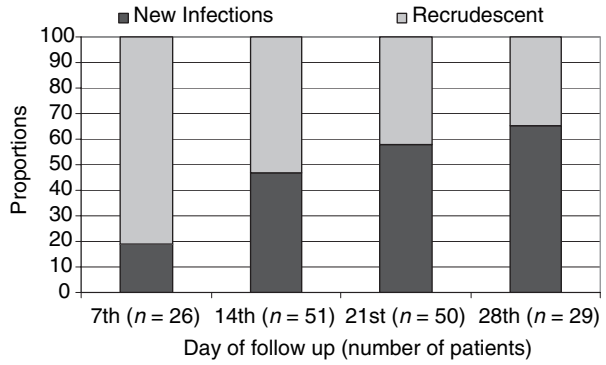

Figure 1 Distribution of PCR-determined recrudescence vs. new infections during follow-up in Ugandan children with uncomplicated falciparum malaria treated with either artesunate or placebo plus standard dose sulphadoxine/pyrimethamine.

over time (i.e. lowest on day 7, highest on day 28) but with high rates on days 7 and 14. The effect of the 29, pre-day 14, new infections is to lower the overall failure rate (all three arms combined) by 7.8% (29/373) by counting unresolved PCR data as failures, or by 8.6% (29/339), if unresolved PCR data are excluded.

Discussion

In the artesunate-based combination therapy trials conducted by WHO/TDR in Sub-Saharan African countries only post-day 14 recurrent parasitaemias were genotyped to distinguish recrudescent from new parasites and treatment failure rates adjusted accordingly. In the present study, we genotyped all recurrent parasitaemias observed on days 7, 14, 21 and 28 from one site, Mbarara, Uganda, in order to assess pre- and post-day 14 re-infection rates. We observed unexpectedly high frequencies of genotypically new infections on days 7 (approx. 19%) and 14 (approx. 47%). Although the entomological inoculation rate was not measured in this study, cumulative re-inoculations during follow-up have resulted in these new infections which represent an important fraction of the total number of recurrent parasitaemias. Given that the prepatent period for *P. falciparum* is 6–12 days, and that pyrimethamine has weak causal prophylactic activity, parasitaemias detected on days 7 or 14 after initial parasite clearance may represent early new infections. Therefore, the assumption that recurrent parasitaemias on days 7 and 14 in high transmission areas are due to recrudescence and should be excluded from genotyping assays may be erroneous. These findings strongly suggest that genotyping should also be conducted on recurrent parasitaemias recorded before and on day 14.

Characterizing these early recurrences may change substantially our appreciation of drug efficacy. By taking these new infections into account, the overall failure rate fell by approximately 7–8%. This change could have a profound effect on deciding drug policy if the efficacy of a given drug is not deemed to have reached a predefined threshold for changing drug policy. The using of PCR-corrected efficacy end points in malarial drug trials has become standard practice but there are variations in the way that the PCR data have been interpreted (Ranford-Cartwright *et al.* 1997; Brockman *et al.* 1999; Basco & Ringwald 2000; Magesa *et al.* 2001; Basco *et al.* 2002; Cattamanchi *et al.* 2003; Happi *et al.* 2004). This calls for a standardized genotyping protocol for areas of intense malaria transmission.

It has been speculated that new infections might already be present during the clinical presentation but sequestered and not detected by PCR only to be picked up at the time of recurrence. Although this might indeed occur in a few cases, ample evidence exists that clinical episodes are less complex. A study by Irion *et al.* (1998) found that <2% of recrudescent genotypes were absent on day 0 but were detected on day 3. Similarly, Farnert and Bjorkman (2005) detected the same genotypes in consecutive samples obtained every 12 h for at least 3 days post-treatment in Swedish non-immune travellers who acquired falciparum malaria in Africa. These observations suggest that single time-point samples may reliably represent all subpopulations present prior to treatment. A further complication in the interpretation of paired genotype data arises from infections with new parasites possessing identical genotypes to those present on day 0, leading to an erroneous diagnosis of recrudescence. However, in endemic areas the probability of this occurrence is rather low and negligible when two or more discriminatory markers are being used. We believe this theoretical possibility is not an important consideration in the interpretation of our findings because we observed a high rate (approx. 33%) of new infections (different genotypes) within the first 14 days of follow-up.

In our study, laboratory failure to amplify/detect parasite genetic material in the recurrent samples was the main cause of the loss of genotyping data. This PCR failure is highly attributable to the low parasite densities in the recurrent samples coupled with a less invasive/rigorous DNA extraction method consisting of simple washing and boiling of the Isocode stix®. The performance and effective use of PCR genotyping will be detailed in a general report on the combination therapy trials that will be published separately.

The definition of drug-resistant malaria and the reporting of drug efficacy data are becoming more complicated

with our better understanding of *P. falciparum* molecular genetics, seasonal variations in malaria transmission, drug pharmacokinetics (e.g. longer follow-up is required for long half-life drugs) and the intrinsic parasiticidal effects of antimalarial drugs (White 1998). Further studies are needed to assess the importance of genotyping recurrent parasitaemias observed between days 7 and 14 and how this might refine the current WHO definitions of late clinical and parasitological failures. More robust definitions will be beneficial to policy makers.

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Disclaimer

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Génotypage moléculaire dans une étude sur le traitement de malaria en Ouganda - taux élevé inattendu de nouvelles infections endéans deux semaines après traitement

Le génotypage des parasites de la malaria par la réaction en chaîne de la polymérase (PCR) dans les études sur l'efficacité des médicaments permet de différencier les réinfections des recrudescences. Un essai comparatif de la thérapeutique de combinaison d'un jour (1AS, n = 115) ou de trois jours (3AS, n = 117) avec de l'artésunate (4 mg/kg/jour) plus du sulfadoxine-pyriméthamine (SP) contre le traitement à base de SP seul (n = 153) a été conduit à Mbarara, une région méso endémique de l'ouest de l'Ouganda. Tous les échantillons appariés de parasitémies récurrentes à *Plasmodium falciparum* aux jours 7, 14, 21 et 28 après traitement ont été génotypés par PCR et analysés pour la protéine riche en glutamate (glurp) et pour les gènes des protéines de surface de mérozoïte (*msp1* et *msp2*) afin de distinguer entre recrudescences et nouvelles infections. 156 (1AS = 61, 3AS = 35, PS seul = 60) sur 199 des échantillons appariés de récurrence ont été entièrement analysés; 79 cas de recrudescences (1AS = 32, 3AS = 8, PS = 39) et 77 cas de nouvelles infections (1AS = 29, 3AS = 27, PS = 21) ont été identifiés. Les rapports des proportions entre nouvelles infections et recrudescences étaient de: 0,2–0,9–1,4 et 1,9 au jours 7, 14, 21, et 28 respectivement (P < 0,001; Chi carré pour le test de linéarité). Des taux élevés inattendus de nouvelles infections ont été observés tôt dans le suivi, aux jours 7 [5/26 (19,2%)] et 14 [24/51 (47,1%)]. Ces résultats ont un impact significatif sur la surveillance de la résistance et soulignent l'importance du génotypage de toutes les infections récurrentes dans les essais avec des antimalariques.

mots-clés: génotypage, recrudescent, réinfections, *plasmodium falciparum*, malaria, thérapie de combinaison, ouganda

Genotipaje molecular en un ensayo de tratamiento para malaria en Uganda – una alta tasa inesperada de nuevas infecciones dentro de las dos semanas después del tratamiento.

El genotipaje de parásitos de malaria mediante la reacción en cadena de la polimerasa (PCR), en ensayos de eficacia de medicamentos, ayuda a diferenciar las reinfecciones de las recrudescencias. Se condujo un ensayo de terapia de combinación de uno (n = 115) o tres (n = 117) días de artesunato (1AS, 3AS 4 mg/kg/día) más sulfadoxina/pirimetamina (SP) versus SP sola (n = 153) en Mbarara, un área mesoendémica de Uganda del oeste. Todos las parasitemias pareadas recurrentes de *Plasmodium falciparum*, en los días 7, 14, 21 y 28 post tratamiento, fueron genotipadas mediante amplificación por PCR y análisis de los genes de la proteína rica en glutamato (GLURP) y las proteínas de superficie del merozoito (MSP) 1 y 2, con el fin de distinguir infecciones recrudescentes de nuevas infecciones. Se analizaron con éxito 156 (1AS = 61, 3AS = 35, SP solo = 60) de 199 muestras pareadas, resolviendo 79 como recrudescencias (1AS = 32, 3AS = 8, SP = 39) y 77 como nuevas infecciones (1AS = 29, 3AS = 27, SP = 21). La razón de proporciones de nuevas infecciones recrudescentes eran 0.2, 0.9, 1.4, y 1.9 en los días 7, 14, 21, y 28, respectivamente (P < 0.001, χ^2 prueba para tendencia linear). Se observaron unas tasas de infección inesperadamente altas durante los primeros días de seguimiento: Día 7 [5/26 (19.2%)] y 14 [24/51 (47.1%)]. Estos resultados impactan significativamente sobre la monitorización de resistencias y muestran el valor del genotipaje de todas las infecciones recurrentes en los ensayos clínicos de antimaláricos.

palabras clave: genotipaje, recrudescencia, re-infecciones, *plasmodium falciparum*, malaria, terapia de combinación, uganda

