The numerical distributions of parasite densities in asymptomatic malaria

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Footnotes

Conflicts of Interest:

None of the authors have any commercial or other association that might pose a conflict of interest

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Abstract

Background:

Asymptomatic parasitemia is common even in areas of low seasonal malaria transmission, but the true proportion of the population infected has not been estimated previously because of the limited sensitivity of available detection methods.

Methods:

Cross-sectional malaria surveys were conducted in areas of low seasonal transmission on the Northwestern Thailand-Myanmar border and in Western Cambodia. Using an ultra-sensitive PCR DNA quantitation method (uPCR: limit of accurate detection 22 parasites/mL) parasite density distributions for *Plasmodium falciparum* and *Plasmodium vivax* were characterised and the proportions of undetected infections imputed.

Results:

The prevalence of asymptomatic malaria by uPCR was 19.9% (988 of 4,975 people tested). Both *P. vivax* and *P. falciparum* density distributions were unimodal and log normally distributed with modal values well within the quantifiable range. The estimated proportions of all parasitemic individuals identified by uPCR were over 70% for *P. falciparum* and over 85% for *P. vivax*. Predicted proportions overall were 83% *P. vivax*, 13% *P. falciparum* and 4% mixed. Geometric mean parasite densities were similar; *P. vivax*: 5,601/mL and *P. falciparum*: 5,158/mL.

Conclusions:

This uPCR method identified most infected individuals in malaria endemic areas. Malaria parasitemias persist in humans at levels which optimise the probability of generating transmissible gametocyte densities without causing illness.
Introduction

Asymptomatic malaria parasitemia is very common in areas of high stable transmission – indeed everyone there may be infected. In contrast asymptomatic parasitemia was considered relatively uncommon in the areas of low seasonal transmission which predominate in much of Asia and the Americas, but recent epidemiological studies using sensitive methods of parasite detection have revised this view (1-8). In Myanmar, Thailand, Cambodia, Laos and Vietnam a substantial proportion of the population living in malaria endemic areas harbor asymptomatic parasitemias (9-11). These infected individuals sustain malaria over the dry season, and so they are an important source of malaria transmission and a major obstacle to elimination. However because of the limited sensitivity of detection methods the proportion of people in any area with chronic low density parasitemias has been unknown. Accurate characterisation of malaria epidemiology and its geographic distribution is essential in planning regional control and elimination strategies. Using an ultra-sensitive qPCR method of parasite detection on blood volumes >200µL [uPCR] (12) the numerical distributions of parasite densities in asymptomatic falciparum and vivax malaria infections were characterised in populations living in malaria endemic regions of Western Cambodia and the Thailand-Myanmar border (11). This method was sufficiently sensitive to identify the majority of infected persons and so allowed prediction of the proportion of the population with parasitemias below the limits of detection with the most sensitive current techniques.

Methods

These studies took place in malaria-endemic regions along the North-West Thailand-Myanmar border and in western Cambodia as a prelude to the assessment of elimination interventions. In these areas malaria transmission is low and seasonal with entomological inoculation rates usually below 3 and often below 1/person/year. Most clinical cases of malaria occur during the rainy season
between May and December (1, 13-18). In the past *P. vivax* and *P. falciparum* each comprised approximately half the clinical cases, although with recent reductions in malaria incidence *P. vivax* now predominates.

Full details of these epidemiological studies have been reported recently (11). In brief, screening surveys were conducted in 73 villages followed by detailed cross sectional malariometric surveys in selected villages. These were four villages located within 10 km of the Thailand-Myanmar border considered representative of the area in terms of environment, ecology, population, and behaviour and three villages in the Pailin region of Western Cambodia selected because they had the highest incidence of clinical falciparum malaria in the village malaria workers’ records from 2012.

**Procedures**

All individuals aged six months or more were invited to participate in the surveys. Individual informed consent was obtained from adults, and parental consent obtained for children under 16 years. Demographic and symptom information was collected from each person and the tympanic temperature, weight, and height were measured. Venous blood (3 mL) was collected from all individuals aged ≥5 years, and 500 µL blood was taken from children aged ≥6 months to 5 years.

Sample processing for the quantitative uPCR included separation of plasma, buffy coat, and packed red blood cells, which were then frozen and stored at -80°C and transported to the laboratory in Bangkok, Thailand for DNA extraction and quantitative uPCR (11, 12). All samples for molecular analyses were handled and processed according to the SOPs developed specifically for these studies (12).
Quantification of malaria parasitemia

Description, evaluation, validation and performance characteristics of the high-volume ultrasensitive qPCR (uPCR) methods have been reported recently in detail (12). In brief the DNA template for Plasmodium PCR detection and quantification was purified from the thawed packed red blood cells. DNA extraction from a carefully measured volume was performed using a QIAamp® Blood Mini kit (QIAGEN, Germany) for sample volumes of packed red blood cells ≤ 200 µL, or a QIAamp® Blood Midi kit for sample volumes between 200 to 2000 µL. The purified DNA was dried completely in a centrifugal vacuum concentrator and then resuspended in PCR grade water. The concentration factor was defined by the original blood volumes (100-2000 uL) divided by the double distilled water volumes (10-50 uL). Two microliters of resuspended DNA was used as template in the qPCR reaction.

The number of parasite genomes in each sample was estimated by an absolute quantitative real-time PCR method using Quanti-Tect Multiplex PCR No ROX® (QIAGEN, Germany). The 18S rRNA-targeting primers and hydrolysis probes are highly specific for Plasmodium species (12). The lower limit of accurate quantitation was 22 parasites/mL of whole blood (12). Quantification of high parasite densities (>30,000,000/mL) using this technique becomes progressively inaccurate.

In uncomplicated falciparum malaria later asexual parasite stages cytoadhere and so are seldom seen in the peripheral circulation, whereas all blood stages circulate in P. vivax infections. P.falciparum parasite density (asexual plus sexual stages) is approximately equivalent to genome density as nuclear division occurs in sequestered not circulating parasites, whereas for P.vivax (which does not sequester significantly) there is one genome per parasite for approximately 36 hours of the 48 hour cycle followed by genome doubling every three hours (Figure 1). Thus in a completely asynchronous P. vivax infection there would be an approximate average of 2.63 genomes per parasite. Both asynchronous and synchronous infections were evaluated.
Species identification

For uPCR positive blood samples malaria parasite species were identified using nested PCR protocols specific to *P. falciparum* (microsatellite marker Pk2), *P. vivax* (microsatellite marker 3.502) and *P. malariae* (18s rRNA) as described previously (12). Where there was insufficient DNA for species identification, or no amplification was obtained in this step, samples were reported as “indeterminate” species.

Statistical analyses

Statistical analysis was performed with Stata 13.1 and R 3.1.1. Description of the complete distribution of *P. falciparum* and *P. vivax* densities required imputation from the measured densities and their respective distributions of the partition of species among the lower density parasitemias in which the species were not identified, and the even lower densities which could not be measured. This was possible because the distributions of measured parasite densities were unimodal with modal values well within the quantifiable range. In this analysis parasitemias >30,000,000/mL were excluded as their uPCR estimation was inaccurate, and parasitemias below 30/mL were excluded to reduce stochasticity close to the uPCR limit of accurate detection. Estimates of the density function for log_{10} parasitemias of *P. falciparum* and *P. vivax* were obtained using the Epanechnikov kernel estimator (Stata command “kdensity”). There were the only parasite species identified in this study so the probability of infection being *P. falciparum* (p_{PF}) (i.e. 1 minus the probability of infection being *P. vivax*) was calculated from the observed proportion of *P. falciparum* infections, assuming that the species proportions were the same in the lower density parasitemias for which the species were indeterminate. Imputed datasets (N=1000) were then created comprising all the observed *P. falciparum* and *P. vivax* cases and the indeterminate cases allocated randomly to species based upon the probability p_{PF}. 
Adjustment to accommodate nuclear division in circulating intraerythrocytic asexual *P. vivax* parasites was examined. It was assumed that there is 1 genome per parasite for the first 36 hours of the 48 hour asexual cycle followed by doubling every three hours until finally there were 16 genomes per parasite in the three hours before schizont rupture. Two scenarios were investigated:

(A) completely asynchronous infections: uniform distribution of parasite ages between 1 and 48 hours following merozoite invasion.

(B) synchronous infections: parasite ages follow a normal distribution with a mean age $\mu$ of 1, 2, 3...

...to 48 hours following merozoite invasion with a standard deviation of 4 hours.

These scenarios correspond to the following adjustment factors to convert genomes to numbers of circulating *P. vivax* parasites:

(A) 0.809 ($= 36/48 + 3/(2*48) + 3/(4*48) + 3/(8*48) + 3/(16*48)$)

(B) $\frac{1}{D} \left[\Phi(36, \mu, 4) - \Phi(0, \mu, 4) + \Phi(39, \mu, 4) - \Phi(36, \mu, 4) + \Phi(42, \mu, 4) - \Phi(39, \mu, 4) + \Phi(45, \mu, 4) - \Phi(42, \mu, 4) + \Phi(48, \mu, 4) - \Phi(45, \mu, 4)\right]$ where $\Phi(x, \mu, 4)$ is the cumulative normal distribution with mean $\mu$ and SD 4 evaluated at value $x$, and $D = (\Phi(48, \mu, 4) - \Phi(0, \mu, 4))$ is the total area under the density function between 0 and 48 hours (Figure 1).

For scenario A, all *P. vivax* parasite genome counts were multiplied by the same adjustment factor to give estimated parasite densities, while for scenario B each parasite count had a randomly assigned mean parasite age value $\mu$ (from the uniformly distributed values 1 to 48) and that count was multiplied by the adjustment factor corresponding to the value of $\mu$ (Figure 1). The adjustments examined represent maximum deviations as they assume all *P. vivax* parasites in the sample are asexual.
For each dataset the following were performed:

(a) Testing using the Kolmogorov-Smirnov test to determine if the log-transformed data did conform to a truncated normal distribution with parameters ($m$; mean and $s$; standard deviation). The truncations correspond approximately to the lower and upper limits of accurate quantitation.

(b) Estimation of the mean $m$ and standard deviation $s$ of the un-truncated distributions of log$_{10}$ transformed parasitemia using the maximum likelihood method.

(c) Estimation of the tail of the distribution corresponding to the lower densities’ truncated segment from the log-normal distribution with parameters ($m$; $s$).

The first 20 imputed datasets were also used to examine the relationship between parasitemia and study site and patient age. A linear model for log$_{10}$ parasitemia with these covariates and random effect for participant to account for multiple measurements was fitted for each imputed dataset. The imputation estimates of the parameter coefficients and their 95% CI were calculated (19). Logistic regression was fitted to estimate relationships between risk of fever and parasite density.

**Ethics Committee approval**

The studies were approved by the Tak Province Community Ethics Advisory Board (CAB), the Cambodian National Ethics Committee for Health Research (0029 NECHR, 04 Mar 2013) and the Oxford Tropical Research Ethics Committee (1015-13, 29 Apr 2013).

**Results**

The 7 villages selected for more detailed studies were 3 Cambodian villages (KL, OK, and PDB) with 1766 inhabitants surveyed between June 2013 and June 2014, and 4 villages along the Thailand-Myanmar border (TOT, TPN, KNH, and HKT) with 2377 inhabitants surveyed between May 2013 and June 2015. Of the population included in the census 83% participated in the surveys in the Cambodian villages and 67% in villages along the Thailand-Myanmar border. The median age of the
participants was 21 years; 37% were under 15 years old and 51% were male. The age distributions were similar in the different villages (11).

**Parasite densities**

In total 4,740 participants were tested on 16,662 occasions with median (range) 5 (1-12) tests per participant. Malaria parasitemia was detected by uPCR testing on 2,432 occasions in 1303 participants: 231 (9.5%) infections were *P. falciparum* only, 1,553 (63.9%) were *P. vivax* only, 52 (2.1%) were mixed and 596 (24.5%) were uPCR positive but there was insufficient DNA to determine the malaria parasite species (“indeterminate”) (Figure 2A).

Distributions fitted to the quantitated parasite densities resulted in exclusion of 122 infections below 30/mL and 31 above 30,000,000/mL (23 *P. falciparum*, 48 *P. vivax*, 3 mixed and 79 indeterminate). The parasite density distributions were lognormal and unimodal (see Figures 2B and 2C for kernel densities). After these exclusions the uPCR measured parasitemias had geometric means (95% CI) of 9,801 (6,238 to 15,399)/mL for *P. falciparum*, 11,919 (10,311 to 13,779)/mL for *P. vivax*, and 476 (406 to 557)/mL for the indeterminate species infections.

Overall *P. falciparum* infections comprised 12.1% (208/1,713) of speciated mono-infections within the detection limits. The proportion of *P. falciparum* infections was 15.3% between parasite densities 30 and 999 /mL and 11.2% with densities ≥1000 /mL (p=0.031). To examine how this affected the estimates of undetected parasitemias the probability of an infection being *P. falciparum* (*p*$_{PF}$) was estimated by (i) method 1: *p*$_{PF}$ = 0.1214 for all densities and (ii) method 2: *p*$_{PF}$ = 0.1534 for densities between 30 and 1,000 /mL and *p*$_{PF}$ = 0.1124 for densities between 1,000 and 30,000,000 /mL.

With the uniform *p*$_{PF}$ = 0.1214, for the 1000 “corrected” datasets, the mean (IQR) of the estimated log density parameters was m =2.86 (2.75 -2.97) with standard deviation s = 2.16 (2.09-2.22) for *P. falciparum* and m = 3.34 (3.33 -3.35) and s = 1.60 (1.60-1.60) with an asynchronous parasite age
distribution for *P. vivax* (scenario A). These estimates were only slightly different for synchronous *P.
vivax* parasite age distributions (scenario B); m = 3.25 (3.23 - 3.27) and s = 1.64 (1.63-1.66)
respectively (Table 1). With the two pfs values (0.1534 and 0.1124) the estimates changed slightly
for *P. falciparum* but not for *P. vivax*; *P. falciparum* mean (IQR) m = 2.64 (2.49 -2.79 ) and s = 2.56
(2.17-2.33); *P. vivax* m = 3.35 (3.35 -3.36) and s =1.59 (1.59-1.60) with scenario A, and m = 3.26 (3.24
-3.28) and s = 1.63 (1.62-1.65) for scenario B, over the 1000 “corrected” datasets. For both methods,
the distribution of the log_{10}-transformed parasite densities in all the “corrected” datasets could be
assumed to be normally distributed (all Kolmogorov-Smirnov tests p> 0.38 for *P. falciparum* and p>
0.092 for *P. vivax*).

After correction for the indeterminate parasitemias, the imputed “corrected” median (IQR)
geometric mean parasite densities for detected infections were 5,158 (4,863 to 5,454)/mL (method
1) and 4,627 (4,382 to 4,940) (method 2) for *P.falciparum*, and 4,529 (4,495 to 4,567)/mL and
4,598 (4,556 – 4,636)/mL for *P.vivax* with scenario A, and 4,454 (4,392 to 4,517)/mL and 4,513
(4,452 – 4,580)/mL for *P.vivax* with scenario B (Figures 3 and 4). The estimated proportions of
undetected infections (i.e. not identified because they were below the limits of accurate
quantitation) were 11.8% (IQR, 11.7-12.0) and 13.6% (IQR, 13.2-14.0) of the *P.vivax* infections for
scenarios A and B, respectively, and 25.6% (IQR, 23.3–27.8) of those with *P.falciparum*. The
proportions truncated at the upper end of the distributions were 0.5% (IQR, 0.5- 0.5) for *P.vivax* for
both scenarios A and B, and 1.6% (IQR, 1.5-1.7) for *P.falciparum*. Taking different pfs ratios above
and below parasite densities of 1000/mL had little effect on the estimates; an estimated 11.5% (IQR,
11.4-11.7) or 13.3% (12.9-13.8) of *P.vivax* infections respectively, and 29.8% (IQR, 26.8-32.7) of
*P.falciparum* infections being undetected. This results in an overall “true” distribution of
asymptomatic malaria infections in these populations as follows; *P.falciparum* 13%, *P.vivax* 83%
mixed 4%.
Parasite densities and clinical covariates

Fever (aural temperature >37.5°C) was detected on 73 occasions in 68 participants who did not complain of symptoms. Participants in Cambodia had higher risk of fever than on the Thailand-Myanmar border (OR 2.44; 95%CI 1.43 - 4.18; p=0.001). The risk of fever increased with parasitemia (OR 1.28; 95%CI 1.08 -1.53 per 10-fold increase; p=0.005) (Figure 5) and was not different between species. Asymptomatic parasite densities were 51.1% (95% imputed CI, 10.4 to 73.4%) lower in children compared to adults, and 31.0% (95% imputed CI, 3.7 to 50.6) lower in Cambodia compared to the Thai-Myanmar border. Overall the differences in parasitemia between P. falciparum and P. vivax malaria were not significant (-10.5%; imputed 95% CI, -41.5% to 36.9).

Discussion

In malaria endemic areas of Western Cambodia and the Thailand-Myanmar border over 85% of asymptomatic P. vivax infections and nearly 75% of asymptomatic P. falciparum infections could be detected by uPCR. The distributions of parasite densities were unimodal and log-normal, as they are for symptomatic malaria, and indeed for many other bloodstream infections. There was no evidence for discrete sub-populations of lower densities. If it is assumed that the malaria species proportions in the lower parasite density range (in which species could not be identified) were consistent with the proportions in the range where species identification was possible then the geometric mean densities for all detected asymptomatic P. falciparum and P. vivax infections were similar at approximately 5 parasites/µL (or 5000/ml, or 2.5 x 10^7/adult).

The uPCR DNA based measurement can overestimate parasite densities slightly, particularly in vivax malaria, as parasite genomes in merozoites in developing schizonts and those free in plasma (less than 0.01% of concomitant intraerythrocytic numbers) and phagocytosed by leukocytes may be quantitated (20). DNA assessment also does not distinguish asexual from sexual parasite stages,
Unlike highly sensitive mRNA methods (21, 22). As gametocytes are cleared more slowly from the peripheral blood than asexual stages, particularly in *P.falciparum* infections, a significant proportion of the parasites detected by uPCR in asymptomatic individuals are likely to be gametocytes. In this study the potential errors related to schizontemia and synchronicity of infection in *P. vivax* infection were examined and found to contribute little to the estimates of geometric mean parasite density or undetected proportions, and measurement errors from free DNA or merozoites were minimized by red cell separation. If the distribution of parasite densities in asymptomatic individuals living in low transmission settings is visualized as an iceberg, only the top of which is revealed by conventional microscopy or rapid diagnostic tests (RDT), then “high volume” uPCR reveals most of the iceberg, and thereby allows prediction of the proportion of individuals who have parasite densities below the level of detection.

These data also suggest that the majority of infected individuals should be detected by the widely used filter paper capillary blood PCR methods which typically sample only 5-10µL of blood (23-25). However the limit of detection for these methods is in the parasite density range which contains a large proportion of asymptomatic parasitemias (1-10 parasites/µL) so small inter or intra-assay changes or variations in assay performance could have large effects on their malaria prevalence estimates.

Asymptomatic infections in low transmission settings may be harbored within an individual for long periods in a quasi-steady state (26-33). If the longitudinal profile of sub-microscopic parasite densities mirrors that in the microscopy countable range, then regular waves of higher densities occur with the sequential emergence of new antigenic variants (27-30, 34). In vivax malaria persistence is enhanced by relapse which occurs in the majority of infections in South-East Asia (35-37). Some of these waves of asexual parasitemia generate transmissible densities of gametocytes (27-30, 33, 34, 38, 39). As at any time the prevalence of asymptomatic malaria is orders of magnitude higher than that of symptomatic malaria the “transmission reservoir” from asymptomatic
malaria is likely to be large. Assuming that a vigorous host response to malaria reduces the probability of onward transmission, particularly in the modern era of antimalarial drug treatment, then from a parasite perspective the optimum density to persist at is one which does not generate illness, but has the greatest chance over time of generating transmissible gametocyte densities.

Are these parasite density distributions from areas of low unstable malaria transmission in Southeast Asia relevant to higher transmission settings where a much larger proportion of asymptomatic individuals have parasitemias detectable by microscopy? Blood stage immunity is greater in higher transmission settings and splenic function is augmented, and thus clearance of parasitized erythrocytes is likely to be more rapid, so the net result of higher inoculation rates will be a truncated lower end of the parasite density range with proportionally fewer densities below the limit of uPCR detection. This is because with a median sporozoite inoculum of approximately 10, and liberation of ~35,000 merozoites from each hepatic schizont, the median blood parasite density following hepatic schizogony of each inoculated brood in an adult (blood volume ~5000mL) approximates 70/mL (which is above the current level of uPCR detection). The greater proportion of very low density *P. falciparum* infections compared with *P. vivax* in this study may reflect the currently lower transmission of this species in the study areas, and thus the greater probability that an individual was being sampled while their infection was being eliminated, whereas *P. vivax* infections were acquired both from inoculation and more frequent relapse (35). In contrast in high transmission settings the upper range of densities of asymptomatic infections is known to be extended as “premunition” elevates the pyrogenic density (27). In such high transmission areas a higher proportion of the population is parasitemic by microscopy or RDT and asymptomatic parasite densities often extend up to 10,000/µL. Age is a greater determinant of parasite density distributions than in lower transmission settings. A recent cross sectional survey conducted in an area of previously high transmission in Papua New Guinea two years after distribution of insecticide treated bed-nets used sensitive nucleic acid based methods of parasite detection and estimated geometric mean parasite densities of 42 genomes/µL for *P. falciparum* and 8/µL for *P. vivax* in identified
infections (21). However densities were substantially higher in young children ($P. falciparum$ ~650 genomes/µL, $P. vivax$ ~140/µL). Overall it is likely that more of the “iceberg” of the parasite density distribution in asymptomatic people will be detectable in the microscopy or RDT detectable ranges in high compared with low transmission settings.

These data suggest that uPCR assessment of parasite densities in population cross sectional surveys provides accurate characterization of the true prevalence of malaria in the community in all transmission settings. If the density distributions reported here do pertain generally (i.e. they are general properties of malaria) then the regional and total global burden of malaria parasites can be calculated, and these data used to estimate accurate selection pressures provided by drugs and other interventions.

**Acknowledgements**

We are very grateful to the communities and their representatives and our many colleagues who helped conduct these surveys.
References


Figure legends

Figure 1. Erythrocytes containing dividing forms of *P. vivax* circulate freely in contrast with *P. falciparum* in which erythrocytes containing dividing forms are sequestered. This correction factor was applied to convert densities of peripheral blood *Plasmodium vivax* genomes to densities of parasites in relation to parasite age.

Figure 2 Part A. Histogram showing parasite densities. Part B. The kernel density function based on the parasite density measurements between 30 and 30,000,000 /mL, estimated for the two species. Part C. The kernel density function based on all parasite density measurements, estimated for each species. The arrows show approximate lower limits of detection for RDTs and capillary blood (5uL) PCR detection.

Figure 3. The predicted geometric distributions of parasite densities for *Plasmodium falciparum* and *Plasmodium vivax*. Results are shown for the mean values of parameters (m,s) for uniform $p_{PF}$: (2.9,2.2) for *Plasmodium falciparum* and (3.3, 1.6) for *Plasmodium vivax*.

Figure 4. Histogram of the imputed parasite densities for *Plasmodium falciparum* and *Plasmodium vivax*. The height of the bars is scaled so that the sum of the heights equals 1. Results are shown for the mean values of parameters (m,s) for uniform $p_{PF}$: (2.9,2.2) for *Plasmodium falciparum* and (3.3, 1.6) for *Plasmodium vivax*.

Figure 5. Relationship between parasite density and risk of fever in this study.
Table 1. Estimated parasite density distributions derived from 1000 imputed datasets.

<table>
<thead>
<tr>
<th></th>
<th>Plasmodium falciparum</th>
<th>Plasmodium vivax – scenario A*</th>
<th>Plasmodium vivax – scenario B*</th>
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<tr>
<td><strong>Predicted log-normal distributions</strong></td>
<td>Mean</td>
<td>Range</td>
<td>IQR range</td>
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<td>standard deviation “s”</td>
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<td>1.90-2.66</td>
<td>2.09-2.22</td>
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<td>% below LLOQ ( &lt;30 /mL)</td>
<td>25.6</td>
<td>16.3–42.2</td>
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<td>% above ULOQ (&gt;30,000,000 /mL)</td>
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* Scenario A: asynchronous infections, Scenario B: synchronous infections – SD 4 hours