Development and optimization of a selective whole-genome amplification to study *Plasmodium ovale* spp.

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**Transaction Report:**

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

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Dr. Valentin Joste
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Re: Spectrum00726-22 (Development and optimisation of a selective whole-genome amplification to study Plasmodium ovale spp.)

Dear Dr. Valentin Joste:

Due to limited availability of reviewers, we were only able to secure one formal review. However, I have evaluated the manuscript and have provided some comments and would like these addressed in the resubmission.

- It would be informative to add a read coverage comparison between non-SWGA and SWGA samples. This would facilitate appreciation of the benefit for using the method.
- Do you know the success rate for clinical sample amplification? Did you encounter any samples that failed? This information is useful for others planning to use this method.
- Do you have recommendations about when to use the various methods? For example, are there sample characteristics that necessitate SWGA vs leukodepletion (perhaps blood volume?).
- How many SNPs are called by both methods? How many overlap between the two methods? These metrics are important because you show in Figure 3 that read count is uneven following SWGA.
- Please provide access to the Perl homemade script used in the phylogenetic analysis.

For Supp Fig 3: there are assumptions when using Pearson correlations- it is important to check that your data fits these assumptions and whether outliers should be removed. For example, outliers should not be included for calculating R².

Minor points:
1. Legend of Supp fig 3: "red points a difference between 50 to 75%" should be "blue points a difference between 50 to 75%"
2. Title: optimisation -> optimization
3. Standardize the format of Supplementary Tables and the name of sheets

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Sincerely,

Jennifer Guler
Editor, Microbiology Spectrum
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1752 N St., NW
Reviewer comments:

Reviewer #1 (Comments for the Author):

The premise of this manuscript is great for malaria biology, where there is limited knowledge on the evolution of other species of human malaria parasites, especially P. malariae and P. ovale. In this manuscript, the authors present a method to specifically amplify the species of P. ovale towards future population genomics analyses that may be informative of its evolution and guide interventions for the complete elimination of all malaria parasites. The method they have presented, though not novel, is applied here to P. ovale species for the first time. There are a number of issues that need to be resolved;

1. The authors should elaborate more on how the background from the human genome and contamination with P. falciparum was assessed in the design of SWGA primers and their use. The only indication is that the SGWA primers for each species were designed, eliminating the other as background. So, were the primer sets specific per species. Can a gel showing no amplification for P. falciparum, humans, and the non-target species be shown? In natural infections, P. ovale is mostly seen as a co-infection with P. falciparum and sometimes with P. malariae too. So eliminating these would help to ensure that these primers can be used in the field.

2. The authors only used suppose monoinfections. Following from the above, these are rare, and larger genomic studies would need to deal with co-infecting Plasmodium species. No evidence on whether the short reads from these monoinfections can map to falciparum or malaria. This control against these other species will be clear evidence of specificity.

3. Controls were leukocyte depleted. It is not clear how these were chosen to be controls. Was SWGA also applied to these controls? To determine the effectiveness of SWGA, SWGA and non-SWGA sequences from the same sample should be compared.

4. For others to use this protocol, it will be helpful to know how much DNA from controls and SWGA was used for library prep, in case these were not amplified. Was the sequencing library prep PCR free or PCR based?

5. It is not clear if the genome coverage report in the main text is for all samples combined and if so, is 10x the mean of median coverage. Did this included coverage for the controls as well?

6. From the scatter plot of parasitemia vs difference between SWGA and McrBc-SWGA, sample IDs could help with clarity.

7. Considering that it is not clear if the short reads generated were mapped against P. falciparum arthologues of drug resistance genes, it is possible that any co-sequenced P. falciparum or P. malaria drug resistance targets would result in variants. As real-time PCR seems to indicate that these were monoinfections, the authors may have to discuss how this will be applied for wild isolates with contaminating coinfections.

8. For the number of samples sequenced, true allele frequencies cannot be determined. If the frequencies reported were from vcftools, then the authors need to indicate that these were determined from read counts and not from consensus data.

9. For the total number of variants detected, the numbers are not clear. For example, 9,782 (3,326 per sample). This seems to be for 3 samples rather than the 5 samples sequenced.

10. Considering there are non-chromosomal contigs for P. ovale species, why were these not also used to map reads. Alternatively, the authors could enrich the manuscript by attempting de-novo assembly.

11. For the interest of the Plasmodium genomics community, genome-wide plots of heterozygosity would be informative, though this will be limited given the sample size.

Overall, the work presented has merits and can be improved. The discussion winds through a repeat of the results, rather than contextualising the outcomes of the work.

Staff Comments:

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- Manuscript: A .DOC version of the revised manuscript
- Figures: Editable, high-resolution, individual figure files are required at revision, TIFF or EPS files are preferred
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Thank you for submitting your paper to Microbiology Spectrum.
Dear Dr. Valentin Joste:

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-It would be informative to add a read coverage comparison between non-SWGA and SWGA samples. This would facilitate appreciation of the benefit for using the method.

Thanks for this suggestion. We did not perform non-sWGA sequencing to prove the benefits of using sWGA. In fact, several publications have already published data about the failure of next generation sequencing without preamplification or filtration (1–4). In the figure below, published by Oyola et al (1), sequencing of Plasmodium falciparum clinical isolates without selective preamplification (corresponding to WGA in the figure) is not possible, with less than 5% of reads that mapped to P. falciparum.

![Image](image-url)

**Figure 1** – sWGA enrichment. from Oyola et al (1).

We considered as unnecessary to test the non-sWGA condition based on those previous observations. On the contrary, we compared the read coverage of the sWGA and leukodepleted samples (considered as controls) to validate the use of sWGA (see figure S3).

-Do you know the success rate for clinical sample amplification? Did you encounter any samples that failed? This information is useful for others planning to use this method.

We did not observe any failure in clinical sample amplification. But we did not test samples with parasite density below 1,790 parasites/µL for P. ovale curtisi and 198 p/µL for P. ovale wallikeri and we cannot predict the success of the amplification below those levels. This is now clearly stated in the discussion lines 405.
- Do you have recommendations about when to use the various methods? For example, are there sample characteristics that necessitate SWGA vs leukodepletion (perhaps blood volume?).

We consider that the best overall technique to amplify *P. ovale* spp clinical samples remains the leukodepletion because it provides homogeneity in reads mapping compared to sWGA. Therefore, leukodepletion allows read distribution-based analyses such as the measure of gene copy number variation (5), related to some drug resistance in *Plasmodium* (6).

In our lab, we used sWGA for samples that we could not filtered in those situations:
- low volume of blood available (minimal red blood cells volume of 200 µL for leukodepletion);
- retrospective study before the implementation of leukodepletion on fresh blood samples;

We add those recommendations in the discussion, line 461 to 463.

- How many SNPs are called by both methods? How many overlap between the two methods? These metrics are important because you show in Figure 3 that read count is uneven following SWGA.

For Poc1, we respectively called 3,732 and 6,980 SNPs with the sWGA and the filtered approaches. 3,638 SNPs overlapped with both methods.
97.4% of SNPs called in sWGA overlap with those from the filtered sample with only 94 SNPs not counted in filtration (64 that did not fit our quality requirement, 8 unmutated positions (0 in coding regions, one NRAF > 0,5 in the sWGA condition) and 22 SNPs with insufficient coverage). On the other side, 52.1% of the SNPs called with the filtration were found in sWGA. The remaining 3,228 positions not detected in sWGA were as follow:
- 1,283 positions that did not fit our quality requirement,
- 1,594 positions with insufficient coverage (but still mainly 0/1 or 1/1) and 409 positions with no coverage,
- 56 unmutated positions (25 in coding regions, NRAF always < 0,5 in filtered condition).

For Pow1, we respectively called 6,045 and 6,125 SNPs with the sWGA and the filtered approaches. 4,793 SNPs overlapped with both methods.
79.3% of SNPs called in sWGA overlap with those from the filtered sample. The remaining 1,252 positions were as follows: 725 positions that did not fit our quality requirement, 394 positions with insufficient coverage and 5 with no coverage, and 128 unmutated positions (49 in coding regions, 1 with NRAF > 0,5).

On the other side, 78.3% of SNPs called with the filtration were found in sWGA. The remaining 1,332 positions were as follows: 696 positions that did not fit our quality requirement, 440 positions with insufficient coverage, 117 positions with no coverage and 79 unmutated positions (18 in coding regions, 2 with NRAF > 0,5).

Please look at figure 2 downside for more details. We add the figure but not the detailed data in the new submitted files and lines 368 to 370 (Figure S6).
-Please provide access to the Perl homemade script used in the phylogenetic analysis.

You will find the Perl script following this link: https://github.com/Rcoppee/P_ovale_sWGA_project. The link has been added in the main text line 481-482.

For Supp Fig 3: there are assumptions when using Pearson correlations- it is important to check that your data fits these assumptions and whether outliers should be removed. For example, outliers should not be included for calculating R2.

You are right, our data did not fit the assumptions of Pearson correlations test after Kolmogorov-Smirnov and Levene test use. We then performed the Spearman rank test to compare the NRAF of both methods.

We ponder the use of R^2 and decided to remove it. In fact, we did not try to establish a mathematical link between the NRAF in sWGA and leukodepletion but only to know if the NRAF (when NRAf > 0) of both methods are correlated.

We completed the statistical part of the method lines 252 to 263.

Minor points:
1. Legend of Supp fig 3: "red points a difference between 50 to 75%" should be "blue points a difference between 50 to 75%"

Thanks for that remark, the modification has been made in the Figure S5.

2. Title: optimisation -> optimization

Thanks for that remark, the modification has been made.
3. Standardize the format of Supplementary Tables and the name of sheets

All supplementary tables have been submitted in one file called Supplemental material except for table S4 and S5 that are too large.

Reviewer comments:

Reviewer #1 (Comments for the Author):

The premise of this manuscript is great for malaria biology, where there is limited knowledge on the evolution of other species of human malaria parasites, especially P. malariae and P. ovale. In this manuscript, the authors present a method to specifically amplify the species of P. ovale towards future population genomics analyses that may be informative of its evolution and guide interventions for the complete elimination of all malaria parasites. The method they have presented, though not novel, is applied here to P. ovale species for the first time. There are a number of issues that need to be resolved;

1. The authors should elaborate more on how the background from the human genome and contamination with P. falciparum was assessed in the design of SWGA primers and their use. The only indication is that the SGWA primers for each species were designed, eliminating the other as background. So, were the primer sets specific per species. Can a gel showing no amplification for P. falciparum, humans, and the non-target species be shown? In natural infections, P. ovale is mostly seen as a co-infection with P. falciparum and sometimes with P. malariae too. So eliminating these would help to ensure that these primers can be used in the field.

When designing primers with the sWGA software, the algorithm asks for a background genome. In our case of Plasmodium blood infection, we chose the human genome as background. We did not provide another Plasmodium genome as background (such as P. falciparum) because only one background genome could be used. The primers’ sets were then not designed as specific to P. ovale spp over the other Plasmodium species but as specific over the human genome. We considered the main issue was the human DNA contamination and not the possible co-infecting Plasmodium species (although this could indeed be another issue).

As you can see in the table below (and added in the new submission, table S2), we calculated the number of binding sites of each primer set on different Plasmodium species as well as the median distance between two primer binding sites.

| Genome size (s) | Poc primers’ set | Pow primers’ set |
|----------------|------------------|-----------------|
|                | Number of binding sites (n) | s/n (bp) | Number of binding sites (n) | s/n (bp) |
| P. ovale spp   | 33 Mbp            | 4,551          | 7,251           | 4,986          | 6,618       |
| P. falciparum 3D7 | 23 Mbp            | 2,127          | 10,814          | 2,267          | 10,146      |
| P. malariae UG01 | 34 Mbp            | 4,218          | 8,061           | 4,791          | 7,097       |
Table 1 - Number of primers’ binding sites (n) and the ratio of genome size/number of binding site (s/n) for the PocGH01 or PowCR01, Pf3D7, PmUG01, PvP01 and GRCh38 genomes for each Poc and Pow primers’ sets.

| Genome          | Size (Mbp) | n   | s/n       | s/n       |
|-----------------|------------|-----|-----------|-----------|
| P. vivax P01    | 29         | 2,760| 10,507    | 2,790     |
| Human           | 2,948      | 23,005| 128,151    | 25,492    |
| P. falciparum   | 10,394     | 115,644|          |           |

The number of primers’ binding is twice larger for P. ovale wallikeri or P. ovale curtisi compared to P. falciparum. Besides, the ratio s/n is twice higher for P. falciparum than P. ovale spp genome. The primers will preferentially bind P. ovale spp DNA over P. falciparum but I’m not sure it will be sufficient when P. falciparum had much higher parasitaemia than P. ovale spp. In our experience in imported malaria with qPCR data and as previously published (7), P. falciparum parasite density is in most cases largely higher than P. ovale spp parasite density (median Ct [IQR] of P. falciparum = 21.5 [18-31]; median Ct of P. ovale spp = 34.5 [29-37]; p=0.003, Mann-Whitney U-test. See (8) for the detailed target of the qPCR).

I have added a paragraph about the sWGA limitations in the discussion lines 441 to 452, notably for Plasmodium co-infections. One of the solution to overcome this issue could be hybridization capture methods (9, 10).

2. The authors only used suppose monoinfections. Following from the above, these are rare, and larger genomic studies would need to deal with co-infecting Plasmodium species. No evidence on whether the short reads from these monoinfections can map to falciparum or malaria. This control against these other species will be clear evidence of specificity.

You are right. To overcome this hypothesis, we concatenate P. ovale curtisi or P. ovale wallikeri genome with P. falciparum genome (Pf3D7, PlasmoDB release 57) and P. malariae genome (PmUG01, PlasmoDB release 57) and aligned P. ovale spp sequencing reads against this new reference genome. As presented as an example on the plot below, P. ovale curtisi (Figure 2A) or P. ovale wallikeri (Figure 2C) reads mapped in large majority to P. ovale spp genome and not to P. falciparum or P. malariae genomes. Besides, reads that mapped to P. falciparum or P. malariae genomes were of poorest quality (Figure 2B and 2D) and of lowest insert size (figure 3A and 3B). Figure 2A to 2D were added to the new submission (figure S2).
Figure 2 – Coverage and mapping quality of \textit{P. ovale curtisi} (Poc1, A and B) and \textit{P. ovale wallikeri} (Pow1, C and D) short reads generated by sWGA against a concatenate genome of \textit{P. malariae} (PmUG01, LT594622 to LT594635), \textit{P. falciparum} (PF3D7, PF3D7_01_v3 to PF3D7_14_v3) and \textit{P. ovale curtisi}/\textit{P. ovale wallikeri} (PocGH01 or PowCR01, LT594582 to LT594595 or LT594505 to LT594518). Plots were generated using Qualimap (v2.2.1)(11).

The insert sizes displayed in the figure 3 represent the part of the paired-end reads that mapped to the reference. The lowest insert size that mapped to \textit{P. falciparum} or \textit{P. malariae} genome (~20 bp, see figure 4) probably represents short consensus sequences between \textit{Plasmodium} species.

Figure 3 – Insert size across reference for A) \textit{P. ovale wallikeri} and B) \textit{P. ovale curtisi}. Plots were generated using Qualimap (11).
Figure 4 – Comparison of *P. ovale curtisi* reads alignment to *P. falciparum* (on the left) or *P. ovale curtisi* (on the right). Gray bases correspond to identical bases as the reference genome. Other colored bases correspond to bases different to the reference genome. Soft clipped bases are bases that are not part of the alignment because of non-identity with the reference genome. Image from IGV (version 2.8.13).

Figure 3 and figure 4 were not submitted with the manuscript and were only made for the response to the reviewers.

3. Controls were leukocyte depleted. It is not clear how these were chosen to be controls. Was SWGA also applied to these controls? To determine the effectiveness of SWGA, SWGA and non-SWGA sequences from the same sample should be compared.

We prospectively chose one *P. ovale curtisi* and one *P. ovale wallikeri* samples received in the French National Malaria Reference Center to be filtered (Poc1 and Pow1). We applied the sWGA to these leukodepleted controls (see table 1, figure 1B) to evaluate the effectiveness of sWGA. We compared the SNPs obtained by the two methods and obtained really closed NRAF (see figure S5).

We did not perform non-sWGA (without leukodepletion) sequencing to prove the benefits of using sWGA. In fact, several publications have already published data about the failure of *Plasmodium* sequencing without preamplification or filtration (1–4). We considered as unnecessary to test the non-sWGA condition based on those previous observations.

4. For others to use this protocol, it will be helpful to know how much DNA from controls and SWGA was used for library prep, in case these were not amplified. Was the sequencing library prep PCR free or PCR based

For the library, 250 ng of DNA was used when possible. For leukodepleted controls, DNA concentration was very low (0.452 ng/µL for Pow1 and 0.114 ng/µL for Poc1) and 50 µL was used (22.6 ng for Pow1 and 5.7 ng). We add those details in the methods section line 187.

The sequencing library protocol was PCR-based.
5. It is not clear if the genome coverage report in the main text is for all samples combined and if so, is 10x the mean of median coverage. Did this include coverage for the controls as well.

The mean coverage reported in the main text (32x for P. ovale curtisi and 24x for P. ovale wallikeri) is for the sWGA method. For the sWGA + McrBC approach, the mean coverage is 62x for P. ovale curtisi and 83x for P. ovale wallikeri. Those results do not include the leukodepleted controls (93x for Poc1 and 99x for Pow1). We also compared between the different methods the percentage of the genome covered with at least 10x (see figure S3b).

6. From the scatter plot of parasitemia vs difference between SWGA and McrBc-SWGA, sample IDs could help with clarity.

We modified the figure with samples IDs.

7. Considering that it is not clear if the short reads generated were mapped against P. falciparum orthologues of drug resistance genes, it is possible that any co-sequenced P. falciparum or P. malaria drug resistance targets would result in variants. As real-time PCR seems to indicate that these were mono-infections, the authors may have to discuss how this will be applied for wild isolates with contaminating coinfections.

Concatenating sequences with P. ovale curtisi or P. ovale wallikeri, P. falciparum and P. malariae resistance genes sequences clearly help to eliminate this hypothesis. We took fastq from previously published P. malariae (ERR4019168 (12)) or P. falciparum (ERR636035 (1)) data and aligned them against the concatenates resistances associated-genes sequences (PF3D7_0417200, PF3D7_0810800, PF3D7_0709000, PF3D7_0523000, PF3D7_1343700, PmUG01_05034700, PmUG01_14045500, PmUG01_01020700, PocGH01_05028400, PocGH01_14036800, PmUG01_10021600, PmUG01_12021200, PocGH01_01016900, PocGH01_10018700 and PocGH01_12019400).
Figure 5 – Coverage across reference and insert size across reference for ERR4019168 on five genes of *P. malariae* or *P. ovale* curtisi orthologous of known resistance genes. Plots were generated using Qualimap (11).

As presented in the figure 5 (for *P. malariae*) and figure 6 (for *P. falciparum*), no *P. ovale curtisi* genes were covered with *P. malariae* or *P. falciparum* reads.

Figures 5 and 6 were not added to the new submission.

8. For the number of samples sequenced, true allele frequencies cannot be determined. If the frequencies reported were from vcftools, then the authors need to indicate that these were determined from read counts and not from consensus data.

I do not calculate allelic frequencies in this study.
9. For the total number of variants detected, the numbers are not clear. For example, 9,782 (3,326 per sample). This seems to be for 3 samples rather than the 5 samples sequenced.

The total number of variants detected is the total of unique SNPs detected, not the sum of the SNPs detected in each sample. If two samples displayed the same SNP, it counts for one and not two SNP. We rephrase the sentences for clarification line 362.

10. Considering there are non-chromosomal contigs for P. ovale species, why were these not also used to map reads. Alternatively, the authors could enrich the manuscript by attempting de-novo assembly.

We actually mapped the reads against chromosomes and contigs of the reference genome and the mapping data we presented are against the whole genome. But we only used the reconstructed chromosomes for SNPs calling because the contigs are mainly composed of pir gene of P. ovale spp. Those pir genes are highly variables (such as var genes in P. falciparum) and is the largest Plasmodium multigene family (13). Due to their high variability among clinical Plasmodium isolates, alignment is not sufficient to obtain high quality data. Local reconstructions, such as previously published for var genes (14), are necessary. No script is actually available to easily reconstruct P. ovale spp pir genes and we decided to not analyze the SNPs obtained on those contigs.

We agree that de-novo assembly would have been of great interest to improve the data. Unfortunately, we do not have the capacity of performing such analysis in our lab.

11. For the interest of the Plasmodium genomics community, genome-wide plots of heterozygosity would be informative, though this will be limited given the sample size.

We add the NRAF profiles as well as the genome-wide plots of the P. ovale spp isolates in the figure S4a and S4b as previously done by Pearson et al (15). Percentage of heterozygote calls were low for both species (<0.1%). We saw less heterozygosity for P. ovale curtisi, maybe linked to the lower depth of coverage compared to P. ovale wallikeri (figure S3 in the Supplemental material).

NRAF plots were quite difficult to interpret in the absence of any reference for P. ovale spp. The heterozygous SNPs we see may be related to background noises due to imperfect reconstructed reference genome or misalignment of reads due to paralogous regions (15). More genomic data are needed to:
- improve the reference genome,
- compute hyperheterozygosity score and used it in the variant filtering approach such as described for P. vivax (15).

12. Overall, the work presented has merits and can be improved. The discussion winds through a repeat of the results, rather than contextualizing the outcomes of the work.

We modified the discussion.

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Dear Dr. Valentin Joste:

We appreciate that the authors addressed all of the reviewer comments in the revised manuscript. Overall, all concerns have been addressed. I am recommending to accept the manuscript, however the data on the cross-mapping of reads at resistance loci (between Pf-Po and Pm-Po) that was included in the response to the reviewers needs to be included and referenced in the final version of the manuscript. This data impacts the use of the method with mixed infection samples, an important application of the method.

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We added the Figure S8 on the cross-mapping of reads at resistance loci between *P. falciparum* and *P. ovale curtisi/P. ovale wallikeri* on one side and between *P. malariae* and *P. ovale curtisi/P. ovale wallikeri* on the other side. We modified consequently the methods section lines 203 to 211 and the results section lines 400 to 403.
August 25, 2022

Dr. Valentin Joste  
French National Malaria Reference Center  
valentinjoste@gmail.com  
Paris  
France

Re: Spectrum00726-22R2 (Development and optimization of a selective whole-genome amplification to study *Plasmodium ovale* spp.)

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