Reviewer’s report

Title: Evaluation of Fluorescent In-Situ Hybridization Technique for Diagnosis of Malaria in Ahero Sub-County Hospital, Kenya 2016

Version: 1 Date: 10 Oct 2017

Reviewer: Jyotsna Shah

Reviewer's report:

PIOverall Comment:

The authors have made an effort to take into account reviewers comments and revise the manuscript. However, there are some major flows with the manuscript which would have to be revised before the manuscript can be published. Based on what is presented, my recommendation is to delete all information on species and concentrate only on Plasmodium genus data by all methods.

Under the study section, there is no mention of the Plasmodium Genus FISH assay. The study section describes only P. falciparum/P. vivax FISH Combo assay. For the Plasmodium genus assay blue filter is required. For the P. falciparum/P. vivax FISH assays two filters are required, one to read green fluorescence (blue filter) and one to read the orange fluorescence (green filter). Only information provided is for the green filter. Secondly for PCR, the authors state that they performed PCR and then confirmed P. falciparum and P. vivax by southern blot. The data they have presented is on Plasmodium genus only. They need to clarify how they confirmed the PCR product as Plasmodium genus. The discussion needs more work. References are wrong. Detailed comments and suggestions are listed below.

Comment 1 Line 31, page 2: microscopy is not feasible [12]. (Boyce & O'Meara 2017).
Reviewer: Delete (Boyce & O'Meara 2017).

Comment 2, 56-57, page 3: The slides were examined microscopically using×100 magnification. Thick blood smears were examined for the presence of parasites while speciation and densities of parasites were obtained by observation of the thin smears.
Reviewer: X100 magnification should be 1000X magnification (100x objective and 10X magnification with eye-piece.) Generally parasite density is calculated using thick smears and speciation by thin smears.
Fluorescence in situ hybridization assays: The FISH assays were conducted on all the samples as per the manufacturer's instructions supplied as part of the P. falciparum/P. vivax FISH combo (ID-FISH Technology Inc., Palo Alto, CA, USA).

Reviewer's Comment: The data on FISH results for Plasmodium genus is presented, however there is no mention of the Plasmodium Genus FISH kit. Please add Plasmodium Genus FISH Kit. Add kit after combo.

Comment 4, Lines 16-18, page 4: The smear was then covered with a cover slip and examined using a fluorescence microscope (magnification: x 1000) fitted with a green filter (excitation and emission of 560 nm and 630 nm respectively).

Reviewer: In the Plasmodium genus test, all Plasmodium parasites are expected to appear green under a blue filter (excitation 492 nm; emission 530 nm band pass). In the P. falciparum/P. vivax combo Test P. falciparum appears green under the blue filter and P. vivax appears orange under the green filter. (excitation 560; emission 630 nm long pass),

Comment 5, Lines 20-24, page 4: Polymerase chain reaction: Briefly, PCR analyses were performed on purified DNA using primers (Mal F2 5' - GCTCTTTCTTGGATTTCTTG -3' and Mal R2 5' - AGCAGGTTAAGATCTCGTG -3'. Following amplification, DNA testing was done by southern dot-blot technique using with P. falciparum and P. vivax dig-probes (5' - AGCAGGTTAAGATCTCGTG -3' and 5' - TCGTCCAATTCTAGAGCAAGC -3' respectively.

Reviewer: The data presented states shows that only Plasmodium genus PCR was performed. No speciation was done. Please clarify how the PCR product was confirmed as Plasmodium genus.

Comment 6, lines 50-51, page 4: Children who were less than 6 months of age constituted 18.2% of the participants while those age between 6 and 24 months were 40.4% of the participants (Table 1).

Reviewer: Delete table 1. Replace above sentence with: Children less than 6 months of age constituted 18.2%; between 6 and 24 months, 40.4% ; between 25 and 59 months, 39.7%; and >60 months, 1.7% of the study participants.
Comment 7, lines 11-12, page 5: Title, Prevalence of malaria, table 2 title: Table 2 - results on malaria prevalence based on diagnostic tests utilized in the study.

Reviewer: What you are describing is your study result. Therefore change the title to Result Summary.

Change the title of Table 2 to: Summary of Plasmodium Genus results based on diagnostic tests utilized in the study.

Comment 8, lines 32-35, page 5: Of the 104 malarial cases detected by RDT, 97 cases (93.3%) were P. falciparum positive. P. falciparum was detected in 67 out of 85 cases which were positive for malaria by FISH method (78.8%). The concurrent malaria parasite positive samples based on FISH and microscopy, FISH and PCR and FISH, microscopy and PCR were 68 (22.5%), 76 (25.2%) and 67 (22.2%), respectively.

Reviewer: The whole manuscript is focused on Plasmodium genus. Providing the species information does not add anything. Delete lines 32-35.

Comment 9, pages 5 and 6: Table 2a and 2b

Reviewer: Tables 2a and 2b can be combined into one table. See below

Comparison of Microscopy, FISH and RDT with Microscopy + PCR as Gold Standard

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity % (95% Confidence Limit)</th>
<th>Specificity % (95% Confidence Limit)</th>
<th>Positive Predictive Value % (95% Confidence Limit)</th>
<th>Negative Predictive Value % (95% Confidence Limit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy</td>
<td>82.2 (72.4-89.2)</td>
<td>100 (97.8-100)</td>
<td>100 (93.9-100)</td>
<td>93 (88.6-95.8)</td>
</tr>
<tr>
<td>FISH</td>
<td>85.6 (76.2-91.8)</td>
<td>96.2 (92.4-98.2)</td>
<td>90.6 (81.8-95.6)</td>
<td>94 (89.7-96.6)</td>
</tr>
<tr>
<td>RDT</td>
<td>91.1 (82.8-95.8)</td>
<td>89.6 (84.5-93.2)</td>
<td>78.8 (69.5-86.0)</td>
<td>96 (91.9-98.1)</td>
</tr>
</tbody>
</table>
Comment 10 lines 34-60, page 6: Table 4
Reviewer: Delete Table 4. It is redundant.

Comment 11, line 14, page 7: African countries [28].
Reviewer: Reference 21 and 28 are the same. Reference 28, is there a typographical error? Did you mean:

Comment 12, line 15-16, page 7: The LED unit has a blue-green filter attached to the regular light microscope with 100X objective. Prior to the advent of LED units, FISH processed smears were read using mercury arc lamp fluorescence microscopes [29].
Reviewer: Add references 30 and 31. The wording needs to be changed to:
It has been demonstrated that the LED unit with a set of blue-green filters, attached to a regular light microscope with 100X objective can be used to read processed FISH smears [21, 28]. Prior to the advent of LED units, FISH processed smears were read using mercury arc lamp fluorescence microscopes [29-31].

Comment 13, line 18, page 7: Reference #30 (…considered a health hazard [30].)
Reviewer: Reference #30 is wrong. It should be Reference#21.

Comment 14, line 25, page 7: Reference #31(gametocytes [31]
Reviewer: Reference 31 is wrong. It should be Reference# 21.

Comment 15, lines 28-33, page 7: FISH detects specific18Sr RNA fragment ----based on morphology only[32].
Reviewer: Please revise: FISH detects specific 18S rRNA fragments in live parasites whereas PCR, RDT and Giemsa microscopy does not distinguish between live and dead parasites. In case of microscopy, parasite detection and speciation is based on morphology only [32], whereas FISH, in addition to speciation, based on presence of specific 18S rRNA sequences, provides morphological information [21, 28].

Comment 16, line 35, page 7: microscopy diagnosis
Reviewer: Add (82%), after microscopy

Comment 18, line 40: GM respectively [28]
Reviewer: Change reference from #28 to #21.

Comment 19, lines 41 -47, page 7:
Research done in western Kenya reported that the P-Genus FISH diagnostic performance was inferior to that of GM. When evaluated using quantitative reverse transcription PCR as the reference method, the sensitivity and specificity of P-Genus FISH was 29.3% and 75.8%, respectively, while GM had 58.2% and 93.0% respectively. GM had a higher predictive values (PPV = 89.8 and NPV = 68.0% respectively) compared to P-Genus FISH (PPV = 56.0 and NPV=50.5%). The lower performance was attributed, at least in part, to the microscopist facing challenges in identifying the Plasmodium rRNA parasites in especially in smears that had high parasitaemia. On the other hand, the P-Genus FISH assay showed a positive capability in identifying very low parasite densities (sensitivity of 90%) [22].

Reviewer: Suggestion: In the present study, 83% (9/11) of the samples between 280 - 1000 parasites/µl of blood and 98% (59/60) samples with >2000 parasites/µl blood were detected by FISH. Also According to Shah et al., the limit of detection for Plasmodium Genus FISH is 170 parasites/µl blood [21]. Therefore, it is interesting that Osoga et al., only detected 42% (22/52) of the samples with >2000 parasites/µl blood and 90% of samples with 2 parasites/µl blood [22].

Comment 20, page 9: Funding: The Research was supported by ---------- Health Laboratories, Kenya.
Reviewer: Please change to: ID-FISH technology Inc, Palo Alto, CA, USA provided the Plasmodium FISH assay kits, for research use only. Research was supported by National Public Health Laboratories, Kenya.

Ease include all comments for the authors in this box rather than uploading your report as an attachment. Please only upload as attachments annotated versions of manuscripts, graphs, supporting materials or other aspects of your report which cannot be included in a text format.

**Are the methods appropriate and well described?**
If not, please specify what is required in your comments to the authors.

No

**Does the work include the necessary controls?**
If not, please specify which controls are required in your comments to the authors.

Yes

**Are the conclusions drawn adequately supported by the data shown?**
If not, please explain in your comments to the authors.

Yes

**Are you able to assess any statistics in the manuscript or would you recommend an additional statistical review?**
If an additional statistical review is recommended, please specify what aspects require further assessment in your comments to the editors.

I am able to assess the statistics

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Please indicate the quality of language in the manuscript:

Not suitable for publication unless extensively edited

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1. No

2. I have stocks in ID-FISH Technology Inc., the company that has developed the Plasmodium FISH test kits. I confirm that this does not alter my adherence to all Journal policies.

3. No

4. No

5. No

6. No

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