Reviewer’s report

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

Version: 0 Date: 08 Aug 2017

Reviewer: Jyotsna Shah

Reviewer's report:

Dear Editor:

I have reviewed the manuscript "Evaluation of Fluorescent In-Situ Hybridization Technique for Diagnosis of Malaria in Ahero Sub-County Hospital, Kenya 2016" by Regina Kandie et al.

The manuscript needs extensive revisions before it can be considered for publication.

Overall concerns

The data as presented is confusing. What are we looking at Plasmodium genus and/or P. falciparum or what? The data as presented does not provide any information. Results should show what is positive at the genus level and also at P. falciparum level by FISH. There is no information on what species were detected by Giemsa, RDT or confirmed by dot-blot on PCR positive samples. This information is critical in determining sensitivity and specificity of the tests.

The Introduction needs to be updated with recent references. RDT is performed on all the samples, however there is no mention of it in the introduction. Current gold standard for malaria diagnosis is Giemsa microscopy.

The method section needs extensive revision.

1. Explanation on why giemsa was performed on Thick and Thin smears. FISH method needs revision. Were all samples tested by Plasmodium genus and P. falciparum FISH tests? What filters were used? What kind of a microscope was used to read slides - fluorescent microscope with mercury lamp or light microscope with LED unit with appropriate filters. What are the expected results. What about controls with each run?

2. RDT - No species information was provided
3. PCR - How was DNA extracted from whole blood? What method was used? PCR details are required - Were the primers and probes designed in-house, if not provide the reference. In the result section what were the species detected?

Result Section

1. For Microscopy, FISH, PCR and RDT species results must be presented. Also a table with number of samples positive by Giemsa at different parasite levels (>10,000; >5000, >1000; >500; >100, <100 detected/µl blood by Giemsa) vs. other methods would be very useful.

2. Determination of sensitivity and specificity; Suggestion It seems appropriate to compare FISH results with Giemsa because in both tests morphological information is provided. PCR and RDTs have limitations e.g. PCR detects DNA. Thus both live and dead parasites are detected. RDT remains positive even after treatment and also gives false negative results as compared to Giemsa, especially early in the disease. PCR should be used for discrepant analysis.

Discussion

1. The authors need to review the 2016-2017 literature on FISH assays, before writing the discussion section.

Detailed critique:

Background

1. The goal of the National Malaria Strategy 2009-2017 is to reduce morbidity and mortality associated with malaria by 30% by the year 2009 and to maintain it through 2015 [7] - Please provide updated 2017 status.

2. Currently, polymerase chain reaction (PCR) assay is also in use and represents the standard method of detecting circulating malaria parasites by detecting parasite DNA through amplification of ribosomal RNA genes. PCR is not a standard diagnostic method. Generally it is used for research or for speciation in reference laboratories. As you have pointed out it is expensive and time consuming. Therefore very few laboratories use it for diagnosis.

3. Though promising, utility of FISH assay technique in diagnosis of malaria is not well established and there is a paucity of information on this. There are several publications on
FISH. Need to discuss them here. RDT test was performed on all samples, however there is no mention of RDT in the introduction. Please include some discussion on RDT.

Method Section

1. Study Section (Under Method Section): RDT, GM, PCR AND FISH - RDT is mentioned for the first time. Please spell it out. (List of abbreviation to be fully spelt out at first reference).

2. Laboratory Procedures: Thick and thin smears - Quantification of malaria parasites was done for positive slides by counting the parasites against 200 white blood cells. Samples were classified as negative if no malaria parasites were observed following examination of 100 microscopic fields.- Please clarify the following: Thick smears were read to determine parasitemia. Thin smears were read to determine species.

3. Rapid diagnostic tests: Blood samples were tested for malaria parasite antigen using SD Bioline Malaria Ag P.f/Pan.(Standard Diagnostics Inc., Korea), RDT kits as outlined in the manufacturers' instructions inserts.- Explain what is detected more clearly.

4. Fluorescence in situ hybridization assays: Which FISH assays were performed? - Plasmodium genus, P. falciparum. Need to clarify kit and Cat. No. Also how did you read the processed smears? Details regarding the microscope and filters is important. What controls were used?

5. Polymerase chain reaction: Briefly, PCR analyses were performed on purified DNA using primers (Mal F2 5’- CGAAAGTGAAGGAGAAG-3’ and Mal R2 5’-TCTCGTTCGAATGTAC-3’). Following amplification, DNA testing was done by southern dot-blot technique using with P. falciparum and P. vivax dig-probes (5’-GTCACCTCGAAAGATGACTT-3’ and 5’-TAAACTCCGAAGAGAATTTC-3’ respectively). - What method was used to extract DNA? Please provide reference to primers and probes used.

Results Section

1. No information as to what was detected by Plasmodium genus FISH and P. falciparum FISHGiemsa, PCR and RDT was provided at the species level

2. Evaluation of the Performance of diagnostic tests: The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the tests are shown in Table 2a. In the present study, Plasmodium sp. infections diagnosed using PCR and/or microscopy were regarded as the "true positives" with the rest being regarded as "true negatives". The
sensitivity and specificity of FISH was, 85.6% and 96.2% respectively, while that of microscopy was 82.2% and 100% respectively. RDT and PCR had sensitivities of, 91.1% and 98.9% respectively. The corresponding specificities were 89.6% and 100%. This is an incorrect way of computing. Since FISH detects live parasites and provides morphological information, it should be compared with Giemsa as gold standard and not PCR or RDT for the following reasons. PCR detects both live and dead parasites. Therefore PCR can be positive even after the infection has cleared. RDT can remain positive even after the infection has cleared. PCR should only be used discrepant analysis.

3. Analysis of the parasitaemia levels in the 74 samples (24.5%) that tested positive for malaria showed that the parasite levels ranged between 280 and 238,440 parasites/µL of blood. The geometric mean malaria parasite density was 19,825 parasites/µL of blood. The majority of samples (73.0%) had high levels of parasitaemia (≥5000 parasites/µl of blood). Those with low and high malaria parasite densities constituted 17.6% and 9.5% respectively of the samples that were positive for malaria parasites. - May be a table showing different levels of parasites with Giemsa as gold standard compared to other methods would be very useful.

4. In particular, the use of light emitting diode (LED) as a source of light makes the technique invaluable in such settings - Please provide reference(s).

5. The P-Genus and PV-FISH assay results were 100% accurate in three sites with overall agreement of 93% being just as high as the present study [25]. - This is not correct. Shah et al. (2015) demonstrated the assay is reproducible.

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.

Unable to assess

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

Yes

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2. Yes. I have stocks in ID-FISH Technology Inc., Palo Alto, CA. The FISH reagent kits used in the study were provided by ID-FISH.

3. No.

4. No.

5. No.

6. No.

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