Global **Malaria** Programme

A WHO external quality assurance scheme for malaria nucleic acid amplification testing

8–9 June 2015, London, United Kingdom

Meeting report

World Health Organization

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1. BACKGROUND

Since 2010, WHO has recommended that every case of suspected malaria be confirmed by a diagnostic test. In 2012, the T3 (Test, Treat, Track) Initiative was launched to encourage malaria-endemic countries, donors and the global malaria community to increase diagnostic testing, treatment and surveillance for malaria. Since then, malaria diagnosis has become an essential component of malaria control strategies. The pivotal role of diagnostic testing for malaria is reinforced by the Global Technical Strategy 2016–2030, adopted at the Sixty-eighth World Health Assembly (A68/28), for universal access to malaria prevention, diagnosis and treatment and accelerated efforts towards elimination. As part of this strategy, new tools and approaches are expected to become available to target the infectious parasite reservoir in humans.

While quality-assured rapid diagnostic tests (RDTs) and microscopy are the primary diagnostic tools for confirmation and management of cases of suspected clinical malaria, their sensitivity is too limited for efficient detection of low-density parasitaemia or sub-microscopic infections. These infections are common in both low- and high-transmission settings and contribute significantly to the residual, continuous transmission of malaria in low-transmission settings.[1] Many nucleic acid amplification tests (NAATs) are available, which provide better diagnostic performance but imperfect reproducibility as compared with RDTs or microscopy for the detection of low-density parasitaemia and uneven reproducibility.

On the basis of the recommendations of the Malaria Policy Advisory Committee in March 2014, WHO issued a policy brief on malaria diagnostics in low-transmission settings (WHO/HTM/GMP/2014.7), recommending that "use of NAA methods in malaria programmes should be considered for epidemiological research and surveys to map sub-microscopic infections in low-transmission areas". In view of the potential role of NAATs in elimination settings, the document indicated that "an international external quality assurance system is strongly recommended to ensure that data obtained from NAA assays are reliable and comparable".[2]

As part of WHO's work in promoting access to quality-assured malaria diagnostic tests, the Global Malaria Programme organized a meeting on preparation of an external quality assurance (EQA) scheme for malaria NAAT on 8–9 June 2015, in London, England, which was attended by 20 participants, including experts in malaria molecular-based testing, representatives of reference laboratories, institutions involved in the quality assurance of diagnostic tests, research institutes and funding partners.

The main objectives of the meeting were to:

- define customer requirements for an NAAT EQA scheme,
- identify the EQA materials and panels needed for an NAAT EQA scheme,
- define the structure of the NAAT EQA scheme,
- identify strategies for resource mobilization and
- reach consensus on the next steps, roles and responsibilities.

2. EXPERIENCE WITH EXTERNAL QUALITY ASSURANCE FOR MALARIA NUCLEIC ACID AMPLIFICATION TESTING

2.1 Controlled Human Malaria Infection (CHMI) trials network

Several research groups routinely conduct trials of controlled human malaria infection (CHMI), usually to evaluate the efficacy of antimalarial drugs or vaccines. In controlled infection, malaria-naive volunteers are infected by mosquito bites or by injection of sporozoites, and the emergence of blood-stage parasites is carefully monitored in order to initiate treatment as early as possible to ensure the safety of the volunteers and avoid or minimize the appearance of symptoms. Efficacy and safety end-points are usually based on microscopy data; however, NAATs are increasingly being used, as they allow earlier detection of blood-stage parasites, thus ensuring participants' safety and data resolution. Several trials have already been conducted exclusively with NAAT-based efficacy and safety end-points. The NAATs used in CHMI laboratories usually target conserved sequences of the 18S rRNA gene.

In order to evaluate the accuracy and comparability of NAATs, several laboratories conducting CHMI trials performed an EQA exercise.[3] Six centres each received a blinded panel of 60 *P. falciparum* samples prepared from a synchronous, ring-stage, high-parasitaemia culture of the 3D7 strain diluted into type A+ whole human blood to concentrations of 300 000 parasites per mL (p/mL), 6000 p/mL, 600 p/mL, 60 p/mL, 6 p/mL and 0 p/mL (negative samples). Aliquots were prepared according to the procedures in place at each laboratory at volumes ranging from 50 µL to 500 µL before being frozen down and transported on dry ice. Each laboratory was given a minimum of 10 samples at each concentration and 10 negative samples, all blinded, for a total of 60 samples. The NAATs at each laboratory achieved a specificity of 100% and limits of detection between 600 and 60 p/mL. Except for the samples with very low parasitaemia, each laboratory found a sensitivity of 96.7–100%. Good agreement (0.5 log10 difference; < 10% CV) was found between measured and expected values, except in one laboratory, where a general quantitative shift was observed and subsequently resolved.

This small-scale pilot EQA exercise showed that the NAATs used in CHMI laboratories produce accurate, comparable data and demonstrated the applicability of an EQA scheme to evaluate the performance of multiple NAAT protocols. To formalize and extend this initial exercise, the laboratories involved suggested creation of the Molecular Malaria Quality Assessment (MolMalQA), a network of reference and partner laboratories to support establishment of an EQA scheme for malaria NAATs. In addition to the anticipated financial resources required, the challenges identified for the scheme include standardization of materials (because of the complexity of the *Plasmodium* life cycle) and accommodation of the variety of tests and source material in use, thus ensuring the compatibility of tests with liquid and dried blood spots and possibly RNA (in addition to DNA) extraction and amplification techniques.

2.2 United Kingdom National External Quality Assessment Service

The United Kingdom National External Quality Assessment Service (UK NEQAS) is a public service that provides EQA schemes for many diseases. According to its website,[4] it comprises a network of 390 schemes operating in 26 centres based in large hospitals, research institutions and universities throughout the United Kingdom. The services cover qualitative and interpretative investigations in reproductive science, cellular pathology, clinical chemistry, genetics, haematology, immunology and microbiology. Each is directed by experts in the field, with support and advice from steering committees.

UK NEQAS Parasitology established a pre-pilot scheme for malaria NAATs in 2014 and presented the results of two pre-pilot with samples distributed to 28 registered malaria laboratories worldwide in which nested PCR or real-time PCR was used. Samples were prepared from infected donors and diluted in blood negative for *Plasmodium* species to concentrations of 20 000 p/mL, 5000 p/mL, 1000 p/mL and 0 p/mL (negative samples), divided into 0.5-mL aliquots and lyophilized. The first roundincluded *P. falciparum* and *P. vivax* species and the second one *P. ovale* and *P. malariae*. Panels containing one sample at each concentration were shipped blind at ambient temperature and reconstituted on site with molecular-grade water. Results were returned from 24 and 25 laboratories during the first and second pre-pilot phases, respectively. Samples could not be shipped to some West African countries during the outbreak of Ebola virus disease, and customs procedures in Brazil and Peru were lengthy and costly. A few false-positive and false-negative results were reported in each study, possibly because of errors in data entry (Figs 1 and 2).

FIG. 1.
Qualitative results in first round of UK NEQAS pre-pilot malaria molecular EQA

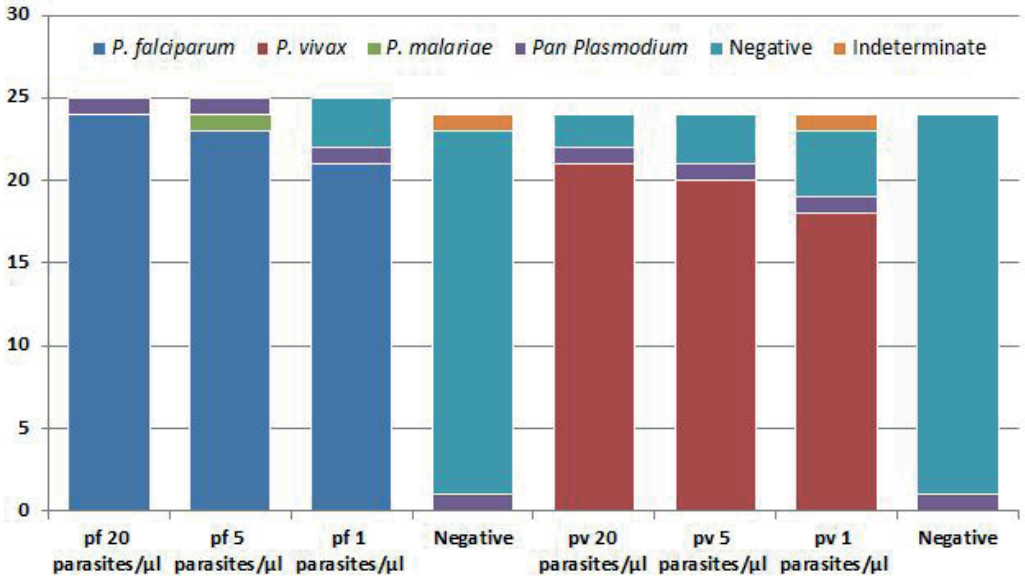
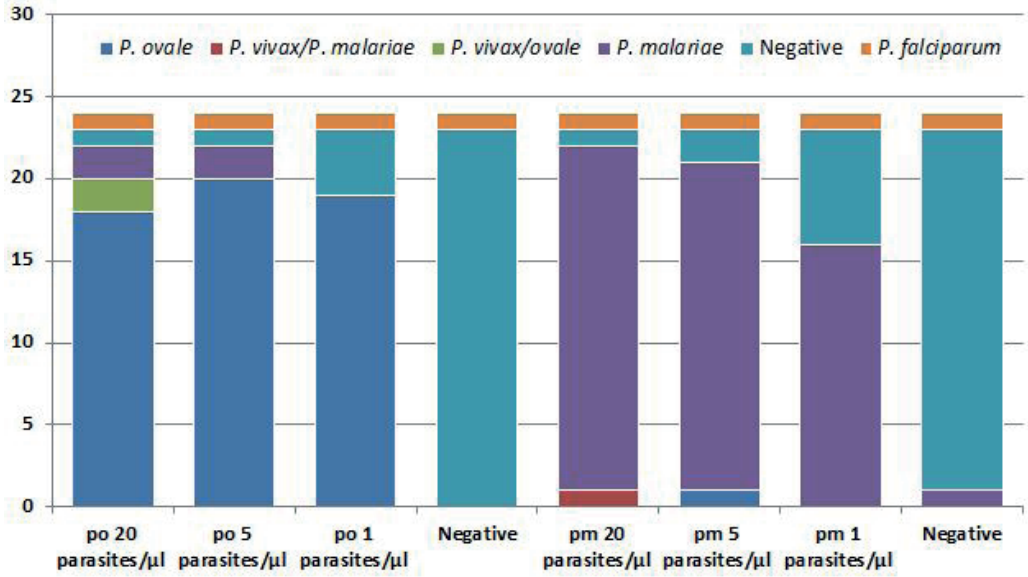


FIG. 2.
Qualitative results in second round of UK NEQAS pre-pilot malaria molecular EQA



Although samples could be ranked by parasitaemia quantitatively, the absolute parasite densities reported were lower than expected. Lyophilization was found to be a straightforward means for reconstitution and DNA extraction and allows transport at ambient temperature. It was concluded that the pre-pilots roundsfulfilled the criteria of EQA, in that they helped participants take individual action to investigate and remedy any discrepant results.

Pilot rounds will be performed in August 2015 and February 2016, with 10 new participating laboratories and the possibility of web-based reporting of results. The full scheme is scheduled to start in April 2016, and an estimate of the costs should be available by September 2015.

2.3 Worldwide Antimalarial Resistance Network

The WorldWide Antimalarial Resistance Network (WWARN) is a global platform for international research on antimalarial drug resistance, Many groups and laboratories face the challenge of comparing data generated on different platforms, with different protocols in different places. WWARN therefore includes quality assurance programmes to improve data comparability and encourage adoption of standard procedures. The quality assurance and quality control programme, which complies with ISO 17043, is a proficiency-testing scheme for pharmacology laboratories involved in studies of the pharmacokinetics and pharmacodynamics of antimalarial agents, which has contributed to improving the performance of the laboratories. An EQA scheme has been set up for molecular typing of *Plasmodium* parasites that are used to differentiate reinfection from recrudescence in clinical trials; 25 laboratories have been participating during the past 5 years, and 7 are enrolled for the next testing round. WWARN has developed a scheme to improve malaria microscopy standards for research and is considering creation of EQA schemes for specific markers of antimalarial resistance.

The results of a quality assurance experiment for the performance of PCR assays in three laboratories and the comparability of the results were presented. The test panel for the experiment was prepared with cell suspensions from the Singapore Immunology Network, containing individually sorted *P. falciparum*-infected red blood cells from ring-stage synchronized culture. The suspension of infected red blood cells was diluted and added to packed cells obtained by depletion of plasma and buffy coat from whole blood to attain equivalent concentrations of 13 852 p/mL, 1385 p/mL, 346 p/mL, 139 p/mL, 69 p/mL, 35 p/mL, 14 p/mL and 7 p/mL. Negative controls containing no parasites and samples spiked with purified *P. vivax* DNA were included in the panel of 112 frozen samples sent to all three laboratories. The results from the three laboratories were in excellent agreement and displayed limits of detection in the range of 16–60 p/mL, well below the pre-defined pass criterion of 100 p/mL.[5] This initial test will be complemented by a further round of tests in November 2015 with samples of concentrations ranging from 10 000 to 5 p/mL in a 96-well format.

It was noted that use of frozen whole blood without RNA stabilizers, as in this experiment, might preclude its use in RNA-detecting NAATs because of the lower stability of RNA; this could be obviated by sorting parasites directly into RNA-stabilizing reagents. Further, the cost of production of these materials is high and may not be appropriate for laboratories that will validate NAATs for routine or clinical diagnosis. Nevertheless, this approach has distinct advantages for ultrasensitive assays (lower limit of detection, ≤ 100 p/mL) used in the detection of submicroscopic infections in research or epidemiological studies or for testing or validating new assays. As individual infected red blood cells are deposited by fluorescence-activated cell sorting (FACS) into specific tubes or wells of multi-well plates, the samples produced with this method minimize Poisson sampling effects, which become significant at very

low parasite densities. FACS-based parasite separation[6] offers the added benefit of separating parasite stages into early rings, trophozoites and schizonts; similar methods for enrichment of gametocytes are being validated. Finally, these samples can be produced in any matrix (whole blood, packed cells with or without RNA stabilizers, frozen, dried spots, etc.) to suit common sampling or assay methods, and the method could also be adapted for *P. knowlesi*, which can be cultured and could potentially be used as an analogue for *P. vivax*. Such quality assurance materials or test panels would be useful for laboratories that wish to certify the performance of ultrasensitive NAATs and for assessing or validating the analytical sensitivity of new assays.

3. ASSESSMENT OF NEEDS FOR EXTERNAL QUALITY ASSESSMENT

3.1 Survey of practices and needs

In order to evaluate current NAAT practices and the perceived need for an international NAAT EQA scheme, the WHO Global Malaria Programme, in collaboration with the University of Washington (USA), designed and conducted an online survey in 2014. Potential participating malaria laboratories were contacted through various communication networks, including WHO regional offices. Responses were obtained from 56 laboratories, most of which are located in Africa and Europe, but also including laboratories in Bangladesh, Brazil, Colombia, India and Japan and other countries in South-East Asia.

The responding laboratories reported that they used various NAATs, including nested PCR (23%), quantitative and qualitative real-time PCR (24% and 15%, respectively), qualitative PCR (15%), reverse-transcription PCR (14%), loop-mediated isothermal amplification (7%) and other NAATs (2%). Half of the respondents were not aware of the existence of a WHO international standard for *P. falciparum* DNA, while 32% (n=9) of those that were aware of it reported that they used it. When asked about the most appropriate sample format for their NAATs, most respondents reported whole blood (40%) and dried blood spots (DBS) (36%); RNA and lyophilized whole blood samples were also used (18% and 6%, respectively). When asked about what samples were considered acceptable, lyophilized whole blood was mentioned by 15% of respondents, probably because they can use such samples even though they usually do not lyophilize samples before processing them. The majority of respondents mentioned whole blood (36%) and DBS (32%) as acceptable samples. Laboratories were not asked whether "acceptable" meant that the sample type had been validated in their laboratory. The target genes reported included 18S rRNA (57%),