TRIBUTE TO DR EAS NELSON - OUTGOING EDITOR OF MALAWI MEDICAL JOURNAL.

Dr Tony Nelson departed at the end of March 1993 to take up a position as Lecturer in Paediatrics in Hong Kong. He had worked as Paediatric Specialist in Kamuzu Central Hospital, Lilongwe, for 4 years, and took over as Editor of this journal in October 1990. Before that time, the journal was known as the Medical Quarterly. With the arrival of a new editor and a new editorial board, the journal changed its name to the Malawi Medical Journal (MMJ).

The journal has flourished under his leadership and guidance. In 1990 there was one edition, in 1991 there were 3 editions, in 1992 there were 3 editions and at the time of his departure 2 editions were already in the pipeline for 1993. In the October 1990 edition, there were 3 original articles in addition to review articles, an editorial and the traditional ward round. In December 1992, there were 5 original articles and 4 short reports. There is now no shortage of articles, case reports and correspondence awaiting publication in the MMJ. It is a tribute to Tony Nelson that he was able to ensure regular publication of a high quality journal, and this has encouraged health workers in Malawi and from overseas to submit their work.

On behalf of the Editorial Board and all the readership of the MMJ, I would like to thank Tony Nelson most sincerely for the hard work, commitment and enthusiasm which he has put in to getting the journal to where it is today. His will be a difficult act to follow, we will miss him sorely, but we wish him and his family all the best in their new venture.

We will try hard to maintain the standard of this journal and ensure regular publication. Dr SB Squire has taken over as Editor and we wish him all the best in his new task.

Professor AD Harries

ORIGINAL ARTICLES

Evaluation Of The Kawamoto Method For Rapid Malaria Diagnosis

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ABSTRACT:
The Kawamoto method for malaria diagnosis uses fluorochrome staining and an interference filter system in a daylight-illuminated microscope. Although suggested to be more sensitive, easier and quicker than Giemsa staining, several problems were encountered when attempting to use the method in a developing country referral hospital. The method in its present form does not appear to be suitable for widespread use in developing countries.

INTRODUCTION

Malaria and its complications are responsible for much morbidity and mortality in Malawi. Blood smear examination for malaria parasites is a major part of laboratory workload and most malaria is treated presumptively. A malaria diagnostic method which is simple, sensitive and cost effective could reduce laboratory workload and reduce the diagnosis of presumptive malaria and hence reduce drug consumption. A new diagnostic technique that is claimed to be more sensitive, easier, and quicker than conventional Giemsa staining has been described (the Kawamoto method) [1]. The technique employs a fluorochrome stain and interference-type filter designed for use with an ordinary daylight-illuminated microscope. The interference filter costs around US$ 46 (1991). Fluorochrome staining allows parasite identification by differential fluorescent emissions from nuclear DNA and cytoplasmic RNA. Normally expensive fluorescent microscopes (halogen-illuminated) are required for fluorochrome staining, which makes the technique unsuitable for much of the developing world.

We evaluated the Kawamoto method of malaria diagnosis at Kamuzu Central Hospital (KCH) in Malawi using documentation and materials kindly supplied by Dr. Kawamoto (one vial of acridine orange (AO) fluorochrome stain (hydrochloride salt) made by Sigma Chemical Co. (cat. no. A4921); Fuji SC-54 barrier filters, and specially designed excitation filters which were funded by the Gashyu Foundation, Gamagouri, Aichi, Japan). The study was undertaken by medical elective students and hospital laboratory staff. The aim of the study was to see if the Kawamoto method could be easily set up in a

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hospital laboratory in a developing country without special training in the technique. This paper describes some of the difficulties encountered.

METHODS

The Kawamoto method can either use a daylight-illuminated microscope or a halogen-illuminated microscope. The fluorescent staining can be done on thin or thick smears. The following summary of the method is taken from documentation supplied by Dr. Kawamoto.

The microscope:

1. Place **barrier filter** into body of microscope (binocular microscope) or eye-piece (monocular microscope).

2. Place **interference filter** below the condenser.

3. Direct flat mirror to sunlight and shield the microscope slide area with black paper. Adjust mirror to direct the blue excitation light to the centre of the microscopic field.

4. Instead of sunlight it is possible to use a halogen light source (slide projector/car headlamp) with the concave mirror of the microscope.

5. For the halogen-illuminated microscope it is necessary to remove all other filters from the microscope and use a paper adaptor to reduce the area of the interference filter exposed to light.

The staining method:

1. THIN SMEAR METHOD - 50 uml of acridine orange (AO) solution (100-150 ug/ml) is put on the cover slip, which is then inverted and placed on the methanol-fixed thin smear.

2. NEW THICK SMEAR METHOD - Mix 5 ul of blood with 10 ul of AO solution, and put on a cover slip.

Malaria parasite nuclei, like those of leucocytes, are expected to fluoresce green. The cytoplasm of the parasite and of lymphocytes fluoresces bright red, and that of other leucocytes a weaker red.

RESULTS

Although the method sounded relatively straight forward we encountered problems at various stages.

MIXING THE AO STAIN

Several attempts to mix the AO stain were needed. The first batch of dye did not appear to emit any fluorescence. Reasons considered were: problems with weighing the dye powder (the weighing balance available at the hospital laboratory was accurate to only 0.1 gm); incorrect pH of solution (There was no pH meter in the hospital laboratory so it was not possible to ensure that the pH of the solution was within the target range of pH 7.0-7.5. There was also uncertainty over how to make up Tris-HCl buffer). A weighing balance (reading to 10^{-4} gms) and a pH meter were subsequently obtained from sources external to the hospital.

The final solution of the AO stain was made up as follows: 0.01 M Tris made up by dissolving 0.1218 gm in 100 ml of distilled water and titrated against 0.01 M HCl to pH 7.4 using a pH meter; 0.0209 gm of AO powder was then dissolved into 100 ml of this 0.01 M Tris-HCl solution, producing an AO concentration of 209 ug/ml (using a target range of 100-300 ug/ml). The solution was then stored in a brown bottle in a dark room.

Smears were prepared using the new thick smear method described above. It was initially noted that the new thick smear method was superior to the thin smear method. Blood that had already been graded as 4+ parasitaemia by the rapid field stain method was initially used. The slide was then examined within a few minutes.

INSTALLING THE FILTER SYSTEM

The next problem encountered was obtaining a standard daylight-illuminated light microscope. In Malawi virtually all district and central hospitals are equipped with electrically-illuminated microscopes. The excitation energy of the electrically-illuminated microscope is not suitable for use with the filter system and the AO stain. A conventional light microscope (Nachet NS200) was eventually located at a nearby teaching institution.

Introduction of the barrier and interference components of the filter system into the microscope presented no difficulties. Observations were made using a x10 ocular lens and x20, x60 and x100 objectives without immersion oil.

EVALUATION OF THE METHOD

Very little illumination was seen with the microscope indoors with the mirror directed to sunlight coming through a window. The microscope was then placed outside in direct sunlight, with the microscope slide area shielded with black paper as advised in the documentation. An orange field was now observed but it was difficult to discern cells and no fluorescent emissions were seen. This was disappointing knowing that the blood sample was rich with malaria parasites, and that the Kawamoto method can detect parasitaemias of less than 0.0002%. An attempt to direct the sun's rays directly to the microscope mirror using a separate free-standing mirror was also tried but did not improve the view.

At this stage it was concluded that problem could be with the stain rather than with the interference filter system. There was some variation in AO stain concentration in the different documentation supplied by Dr. Kawamoto. The first report used 10 - 50 ug/ml AO in 0.05 M Tris-HCl, pH 7.4 and stated that AO concentrations higher than 100 ug/ml were inadequate [1]. The second paper recommended AO staining at a concentration of 100 ug/ml when using daylight-illuminated microscopes [2] and in other documentation a concentration of 100 - 300 ug/ml of AO stain were recommended for the "new thick smear" method.

It was stated that "quenching" of fluorescence may occur under strong Ultra Violet light. To test the activity

of the dye some dry AO powder was placed on a slide and viewed with a Leitz SM-LUX fluorescent microscope available at the KCH laboratory. The dye was seen to fluoresce strongly. More smears were made using the "new thick smear method" with blood graded as negative or 3+/4+ parasitaemia on the rapid field stain method. The smears were then examined using the Leitz SM-LUX fluorescent microscope (filter number 1). For the first time definite fluorescent emissions were seen. However, despite colour pictures supplied with the documentation, there was some uncertainty over interpretation of the fluorescent emissions. In a blinded fashion 5 of the 6 positive samples were correctly identified as were 3 negative samples.

It was now clear that the AO stain was active. The inference filter system in the light microscope was retried but this time a halogen light source was used (slide projector). A new improved barrier filter had also been supplied by Dr. Kawamoto in the meantime. A further 11 slides were prepared using the "new thick smear method". These samples included blood with no malaria parasites, 1+, 2+, 3+, and 4+ parasitaemias. Smears were coded and examined blind. Fluorescent emissions were now detected with the interference filter system but the intensity of the emissions observed with this arrangement were significantly less than that seen with the fluorescent microscope. In none of the 11 samples (5 of which were positive for malaria on the rapid field stain) could emissions from malaria parasites be confidently identified. The same 11 smears were also examined with the Leitz fluorescent microscope but now only 4 out of the 11 samples were graded in agreement with the rapid field stain method.

At this stage it appeared that the AO stain was active and that the inference filter system with a halogen light source was functional. The main problem now appeared to lie with the inexperience of the observers in knowing what to look for. It was also unknown as to whether interference filter system had been optimally set up.

DISCUSSION

We were thus unable to confidently diagnose malaria using the Kawamoto method of rapid malaria diagnosis. There were several reasons why we failed. There were initial problems with mixing up the AO stain. The interference filter system seemed easy to insert into the daylight-illuminated light microscope but with normal daylight no fluorescent emissions could be seen. Using a halogen light source, indistinct fluorescent emissions were seen but there was difficulty interpreting what was seen.

Kawamoto states that the AO staining method with the interference filter is superior to conventional Giemsa staining. Yet, because it requires a drying time of 30 minutes, Giemsa is not used routinely in Malawi for malaria diagnosis. Instead, the rapid field stain is preferred because it takes very little time to prepare and is easy to do. It would therefore be important to show that the Kawamoto method is superior to the rapid field stain method when done by someone experienced in both methods.

Practical problems included lack of conventional daylight-illuminated light microscopes. Even if widely available there would be the problem of not being able to use the method during the night or during much of the wet season when there is a lot of cloud-cover. In addition slide projectors are a rare commodity in many developing country hospitals.

CONCLUSION

The Kawamoto method was not as straight forward as it initially appeared. This will limit its potential for widespread use the developing world. It would appear necessary to supply ready-made AO solution. At present there are too many variables in preparing the stain and in setting up the microscope to guarantee that the desired results will be obtained. Someone familiar with the method would be required initially to oversee preparation of the AO stain, to supervise filter insertion, and to instruct on the interpretation of the observations.

Subsequent discussion with Dr. Kawamoto indicated that the best concentration of AO stain is 50 - 100 ug/ml and that the use of thin smears rather than thick smears is now recommended. It also appears that the experience of other researchers has shown that an external halogen light source is preferable to sunlight. However slide projectors and other halogen light sources will not be readily available in developing countries.

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