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☐ **Brief Communication** ☐

Comparison of acridine orange and giemsa stains for malaria diagnosis

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Abstract: Recently, the Acridine orange (AO) staining method has improved for identification of malaria parasites. Fixed and preserved blood smears of malaria patients were used for comparative analysis of AO and Giemsa stains. The AO staining method required less time and was more sensitive under lower magnification than the Giemsa staining method. The AO staining method provides an alternative to Giemsa for malaria diagnosis in the field and laboratory.

Key words: Malaria diagnosis, Acridine orange, Giemsa stain, comparison

Human malaria results from the infection by one of four species of the genus *Plasmodium* and is characterized by hemolysis of infected red blood cells (RBC) and paroxysmal fevers. Malaria is considered as one of the most important infectious diseases with more than one million deaths among 100 million people infected each year (WHO 1992). In Korea, *P. vivax* had been eradicated since 1984 when only two autochthonous cases were reported (Soh *et al.*, 1985). Recently, however, autochthonous cases of the vivax malaria have been reported in Korean soldiers and civilians residing in the northern part of Kyonggi-do (Chai *et al.*, 1993; Cho *et al.*, 1994; Kho *et al.*, 1994). This year, the authors also diagnosed an autochthonous case of the tertian malaria from a retired soldier from the ROK army previously assigned to Yonchon-gun, Kyonggi-do. Moreover, imported cases of malaria are also increasing due to frequent travel of Koreans to malaria endemic areas (Lee, 1989).

The definitive diagnosis of malaria depends on the demonstration of malaria parasites in stained blood smears. The Giemsa staining method is recognized as the diagnostic method of choice. However, light microscopic observation of the Giemsa stained blood smears is labor-intensive and time-consuming for the diagnosis of malaria. Additionally, extended periods of observation are required to detect low levels of parasitemias which frequently occur in the early phase of the infections, relapsed cases, or chronic infections (Spielman *et al.*, 1988; Rickman *et al.*, 1989; Wongsrishanalai *et al.*, 1991). In the case of the falciparum malaria, delayed diagnosis may result in fatal outcomes or severe sequeles (Rickman *et al.*, 1989). More sensitive and timely methods are needed, not only for the diagnosis of those cases, but also for the mass surveys.

The indirect fluorescent antibody technique was applied to improve diagnostic sensitivity (Collins & Skinner, 1972). The technique is unable to distinguish past infections from present diseases. The technique is now used primarily for mass surveys rather than for clinical diagnosis of malaria. The diagnostic potential of automated differential instruments for malaria or babesiosis diagnosis was evaluated, but the sensitivity was too low to detect low levels of parasitemia (Bruckner *et al.*, 1985; Garcia *et al.*, 1986). McLaughlin *et*

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al. (1991) reported that dot blot assay using a nonisotopic DNA probe provides significant improvements over other DNA based procedures previously reported (Barker *et al.*, 1986). Because of relative cost of the reagents used in DNA probe assays, less expensive and simpler diagnostic methods are needed.

Acridine orange (AO), a fluorescent dye, binds with nucleic acids and fluoresces. The fluorescence can easily be observed in contrast to the dark background. DNA bound with AO fluoresces a bright yellow or apple green while RNA fluoresces orange (Sodeman, 1970). The AO stain, originally combined with microhematocrit centrifugation using Quantitative Buffy Coat (QBC) tubes, was regarded as an alternative to the Giemsa stain (Long *et al.*, 1991; Baird *et al.*, 1992). The principal advantages of the original QBC method were sensitivity, rapidity of staining, and ease to interpret. The disadvantage of the method was the requirement for a fluorescent microscope with 0.3 mm or greater working distance of objective lenses, and the QBC microcentrifuge, which are relatively expensive.

A simple modification in which the AO stain is placed directly onto slides with blood-films, makes the AO method less costly and more timely (Kawamoto, 1991). With the modified method, a standard light microscope with an interference filter can substitute for a fluorescent microscope, reducing cost significantly. A centrifuge, required for preparation of QBC tubes, is also unnecessary. Therefore, this modified method can be applied more easily in field laboratories with limited budgets.

The present authors compared the Giemsa and the AO staining technique for malaria diagniosis. Fixed blood smears of malaria patients were used to compare the efficacy of Giemsa stain and AO staining technique for malaria diagnosis. One patient was infected in the Solomon Islands while the other was an autochthonous case of the vivax malaria from Yonchon-gun, Kyonggi-do, Korea.

Slides with blood smears were prepared for the AO staining by fixing them in absolute methanol for 1 minute, air-dried, and added with 1-2 drops of 1% AO in 0.1 N Tris-HCl, and then observed directly with a fluorescent microscope. For the microscopic observation of intraerythrocytic malaria parasites of Giemsa stained slides, an oil emulsion objective lens (× 100) was required, but the dry high lens (× 50) was sufficient for the AO method.

Fig. 1 shows intraerythrocytic malaria parasites of various stages stained with either Giemsa stain or AO. The nucleus of *Plasmodium vivax* parasites stained red with Giemsa stain and bright yellow with the AO stain. The cytoplasm of the vivax malaria parasites stained dark violet with Giemsa stain and orange with AO. Applique forms, trophozoites of very early intraerythrocytic infections, are shown in Fig. 1A (a & b). RBC infected with more than one trophozoite was also observed (B). Early trophozoites, ring forms, in infected RBCs are presented in Fig. 1C (a & b). Late stage trophozoites with fimbriated edges and trophozoites with double dots are shown in Fig. 1D and E (a & b). An infected reticulocyte with the fluorescent dots in its cytoplasm was easily observed on AO stained slides. A mature macrogametocyte of *P. vivax* stained with Giemsa stain is oval and nearly filling the red cell (Fig. 1G). Mature schizonts stained with Giemsa stain have numerous dark violet colored dots while those stained with AO are bright yellow (Fig. 1H a & b).

The sensitivity and rapidity of diagnosis with the improved AO methods were uncomparably high in the present study. The specificity was also sufficient to differentiate between intraerythrocytic stages of *P. vivax*. Thus, this method can be applied in mass surveys of malaria as well.

Autochthonous cases of the vivax malaria recently reported in Korea indicate the necessity for mass surveys to identify the reservoirs. One of the patients, whose blood was examined in the present study, had presumably become infected in Kyonggi-do where most of recent autochthonous malaria cases are reported (Kho *et al.*, 1994). The other case was imported from the Solomon Islands where both vivax and falciparum malaria are known to be endemic.

Larger sample population should be screened for further survey. Based on limited data, application of the AO staining is strongly recommended for malaria diagnosis of clinical cases and mass surveys.

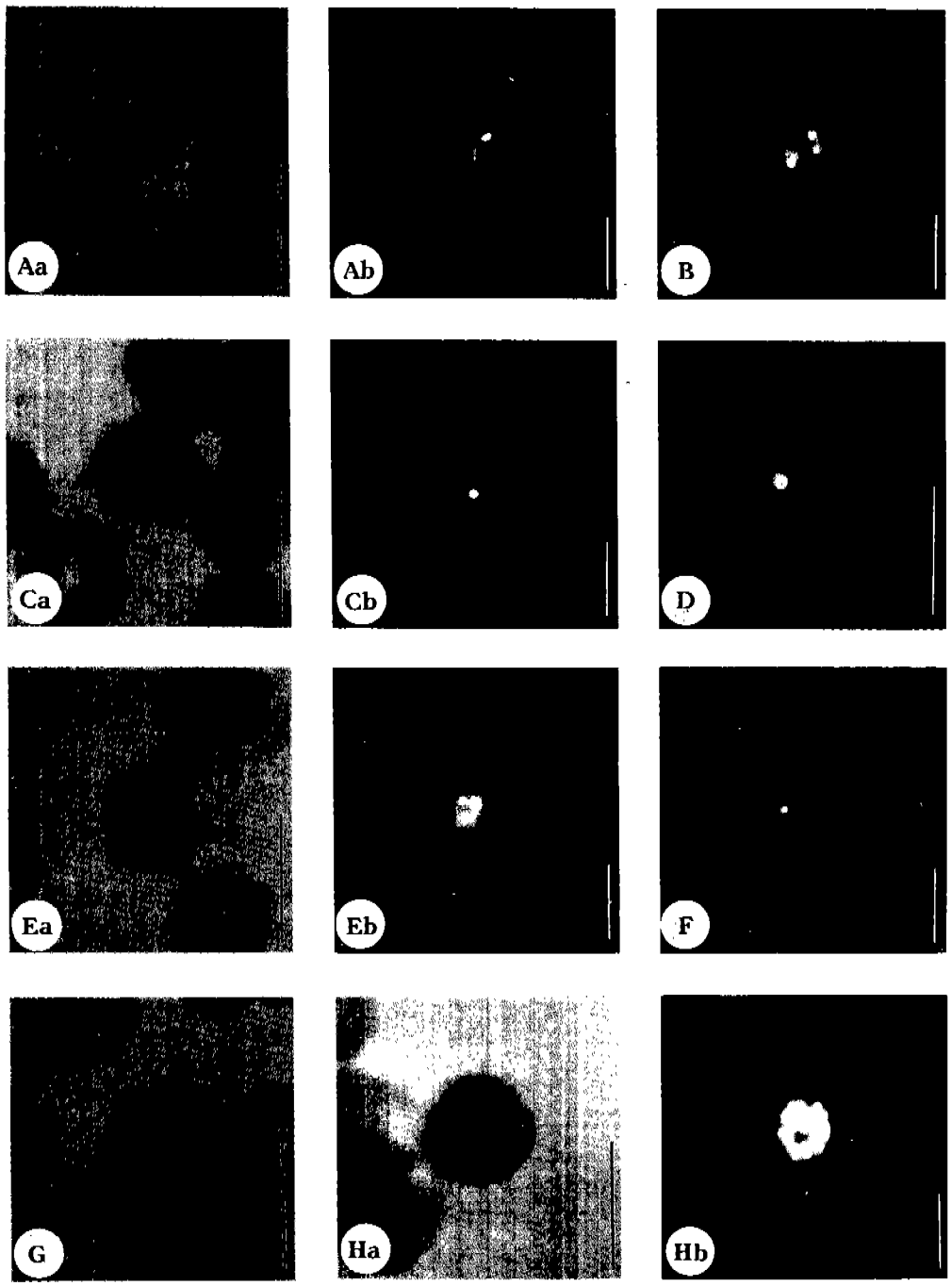

Fig. 1. Intraerythrocytic malaria parasites. **A.** applique form, (a) Giemsa stain, (b) AO; **B.** double infection, AO; **C.** early trophozoite (ring form), (a) Giemsa stain, (b) AO: **D.** late trophozoite, AO; **E.** double dots, (a) Giemsa stain, (b) AO; **F.** trophozoites in reticulocyte, AO; **G.** *P. vivax* gametocyte, Giemsa stain; **H.** schizont, (a) Giemsa stain, (b) AO; Bars indicate 10 μm.

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=초록=

말라리아 진단을 위한 Acridine Orange 염색법과 Giemsa 염색법의 효율성 비교

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말라리아의 진단 방법으로 흔히 사용되고 있는 Giemsa 염색법과 형광염색법중 Acridine orange(AO) 염색법을 비교하였다. 말라리아 환자의 혈액을 채취하여 Giemsa와 AO로 염색하여 각각 광학현미경 및 형광현미경으로 관찰하였다. AO 염색법은 Giemsa 염색법에 비해 저배율에서도 쉽게 말라리아 원충을 찾을 수 있어 빠르고 정확한 말라리아의 진단을 위해 Acridine Orange 염색법이 더 효과적이라고 생각된다.

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