

Full Length Research Paper

Light microscopic detection of *Plasmodium falciparum* *in vitro* through *Pf* histidine rich protein 2 (HRP 2) gold conjugate labeling: Rapid diagnosis of cerebral malaria in humans

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Plasmodium falciparum (*Pf*) has been found to be the deadliest of all the known species of the parasite capable of infecting humans; this is because it is capable of causing severe cerebral tissue damage. This study was carried out to demonstrate the parasite in the host blood *in vitro* through immunogold labeling using antibodies against *Plasmodium falciparum* histidine rich protein 2 (HRP 2); a major metabolite released during the cause of the parasite infection and feeding in the erythrocyte. 12 known *Pf* positive samples were obtained from across the six geopolitical zones of Nigeria and were further characterized by Geimsa thick and thin film for parasite identification parasite count expressed as parasites/ μ l of blood. An average of 400 parasites/ μ l of blood was obtained in each of the samples used for this study. *Pf*-HRP 2 antibody was conjugated to freshly prepared colloidal gold of particle size 40 nm. The conjugation process was blocked with bovine serum albumin (BSA) and the conjugate itself preserved by 1% glycerol and 0.01% sodium azide. The parasite count was titrated against the *Pf*-HRP 2 gold conjugate and was analyzed under the light microscope with a fluorescent filter. Reactivity and specificity of *Pf*-HRP 2 gold conjugate was found to be highly specific and gave direct identification of the erythrocytes infected with the parasite. A good contrast was also obtained between uninfected erythrocytes, parasite and the infected erythrocytes.

Key words: *Plasmodium falciparum*, malaria, fluorescent microscope, HRP 2, antibody, gold conjugates.

INTRODUCTION

Malaria is a dominant infection in the tropics, especially in Africa, South East Asia and the Amazon of South

America, this so because these regions provide suitable climate conditions for the vector to survive and reproduce

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Abbreviation: *Pf* -HRP 2, *Plasmodium falciparum* histidine rich protein 2.

(the vector being female anopheles mosquito) (Fukuda et al., 2009). The parasite in the host system binds to the endothelium of cerebral vessels in the brain; the binding process is called cyto-adherence and it involves knob formation (Mayer et al., 2012). Accumulation of infected erythrocyte in these blood vessels thus prevents exchange of nutrients in the capillary bed, leading to a state of ischemia or anoxia (Hora et al., 2009; Nacer et al., 2011). The tissues of the brain are supplied in segmented tissue blocks of about 1 mm³, such that a tissue block will correspond to the region solely supplied by a terminal artery. Terminal arteries are end arteries that do not branch or anastomose; therefore shortage of blood supply cannot be compensated for eventually causing avascular necrosis primary to cerebral symptoms and hemorrhage (Willie et al., 2012).

In view of this, rapid diagnosis of falciparum infection is important. To detect plasmodium falciparum and other species of the parasite, several proteins have been identified as suitable indicators of the presence of such parasite in the blood stream (Hansen et al., 2012). Examples of such proteins include plasmodium aldolase, plasmodium lactate dehydrogenase and plasmodium histidine rich protein 2 (HRP 2) (Waisberg et al., 2012). Histidine rich protein 2 has been described as being the most effective in detecting falciparum infection (Grobush et al., 2003). A major challenge of rapid diagnostics using *in vitro* kits is that the HRP 2 remains in the blood stream after the parasite has cleared post treatment with an anti-malaria molecule, thus giving false results (Grobush et al., 2003). The World health organization (WHO) recommends microscopy as the standard for diagnosis of malaria and identification of parasite because of the various limitations of the rapid diagnostic tests (RDTs) (prozone effects, false positive and cross reactions) (Cojoc et al., 2012). In this technique we have brought together in the same context the principle of microscopy and the biological techniques of *in vitro* test kits as a fast method in which *Pf* infected parasites can be quickly identified. This method utilizes the microscopic properties of nanoparticles (colloidal gold) and its ability to serve as a fluorescent agent and a tool for microscopy.

MATERIALS AND METHODS

Preparation of colloidal gold and gold conjugate

1% sodium citrate solution was made by dissolving 1 g of sodium citrate (Sigma) in 100 ml of deionised water (Millipore, France) the water was tested for salts and have a pH of 6.0. 2000 ml of the water was placed in a conical flask and 50 ml of 1% gold (99% pure from Sigma) chloride was added. A stirrer magnet covered with a teflon was inserted into the solution, the hot plate was switched on such that the temperature was set at 70°C, a conical flask, 250 ml capacity was inverted to cover the opening of the larger conical flask in order to prevent loss of water by vaporization. The solution was allowed to boil and the stop watch was started, until the colour

changes from red to purple (approximately 1 min). 9.6 ml of 1% gold chloride was added and the colour again changed from red to purple, 2 ml 0.7% gold chloride was then added as the colour was observed to have changed from purple to pink. The solution was allowed to cool by constantly running water on the cone region of the conical flask. The colloidal gold was analyzed using the spectrophotometer in the spectrum region, and the peak absorbance recorded was 528 nm. The particle size as analyzed under the fluorescent microscope was approximately 40 ± 1 nm, modifying the methods of Han et al. (2012).

Conjugation of gold with *Pf*-HRP 2 antibody (monoclonal)

The Tris HCl was poured into a beaker and the antibody was added at a concentration of 5 mg/L, the colloidal gold, twice the volume of the buffer, was added and the solution was blocked with 1.5% bovine serum albumin (BSA, Sigma), added as 10% solution, 0.05% sodium azide (Sigma) was added to prevent microbial infection and 1% glycerol was then added to preserve the conjugate. The solution was centrifuged at 10000 rpm and 4°C, the supernatant was collected and discarded while the final conjugate particle size and concentration was determined, using the spectrophotometer. The colloidal gold and gold conjugate samples were profiled in a hollow slide under the fluorescent microscope (Han et al., 2012; Tsai et al., 2012).

Incubation of gold conjugates and blood samples

100 µl of the gold conjugate was obtained in an eppendorf and mixed with 10 µl of the sample. The samples were mixed by gently shaking the eppendorf tube, and was incubated at 37°C temperature for 15 min, 0.05% of phytohemagglutinin (PHA) was added to partially lyse the red blood cell (RBC). The mixture was then smeared on a hollow slide and viewed under the fluorescent microscope and compared with a normal smear of *Pf*-HRP 2 conjugate. The gold conjugate was also used in soaking a conjugate pad and assembled into a rapid diagnostic kit on a nitrocellulose membrane coated with anti HRP 2 antibody to allow for a double sandwich immunoassay.

RESULTS AND DISCUSSION

The colloidal gold particles can be seen as dots (otherwise quantum dots) in the light microscope while the curves of the spheres can give fluorescence with a filter in place (green fluorescence filters were used in Zeiss Primo Star light microscope, magnification ×1000) (Figures 1A and B). Arrow heads in Figure 2A shows the symmetrical attachment of the tail of the Y shaped antibody leaving the epitope regions (the free ends of the Y) directed at right angles from the surface of the spheres. In this study, approximately four antibody projections were observed per colloidal gold in focus. Arrow heads in B shows the Y shaped slender free ends of the antibodies (magnification ×1000). In Figure 3, colloidal gold (c), parasite (p) and labeled parasite (f) were embedded in the erythrocyte. F1 represents an advanced stage of parasite infection while F2 represents the late

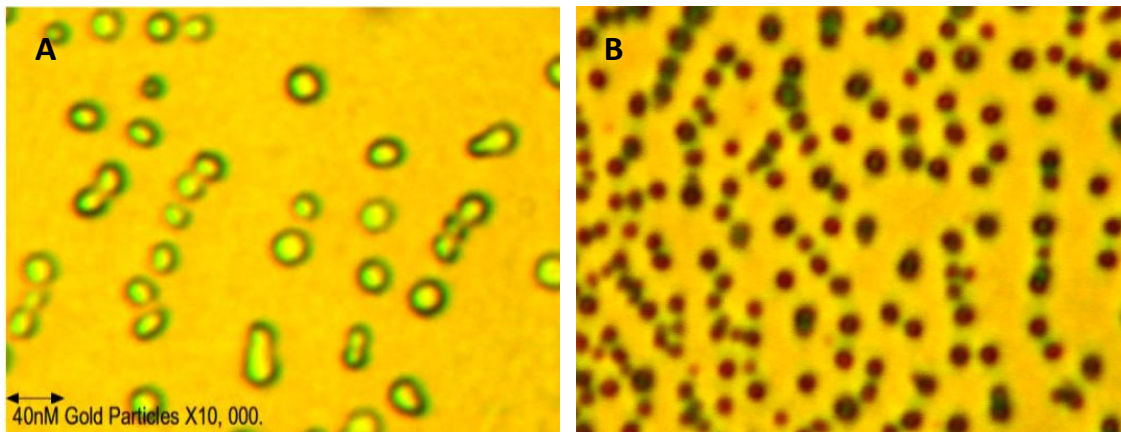


Figure 1. 40 nm colloidal gold under light microscope with fluorescence filters, this is to demonstrate in the first instance that colloidal gold can be viewed without enhancement under the light microscope (B), and with fluorescence filters in the same microscope in (A) (magnification $\times 1000$).

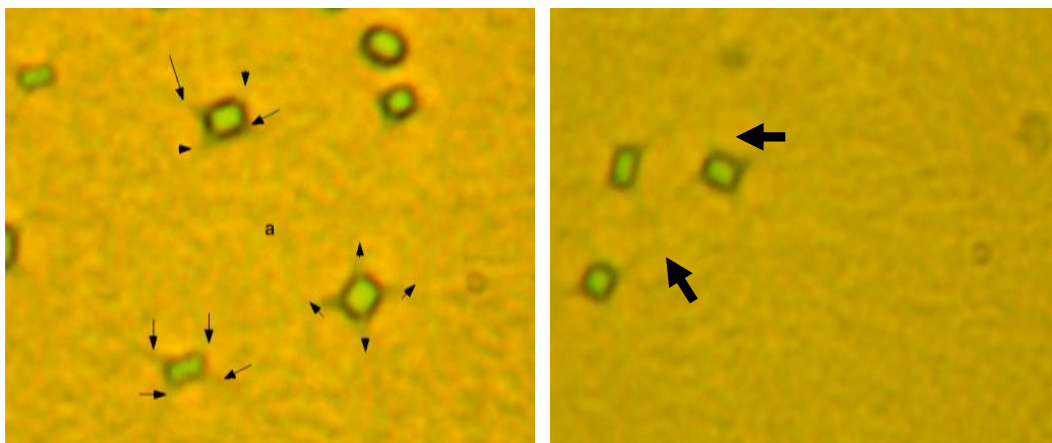


Figure 2. Demonstration of colloidal gold conjugated with *Pf*-HRP 2 using the fluorescence filters (magnification $\times 1000$).

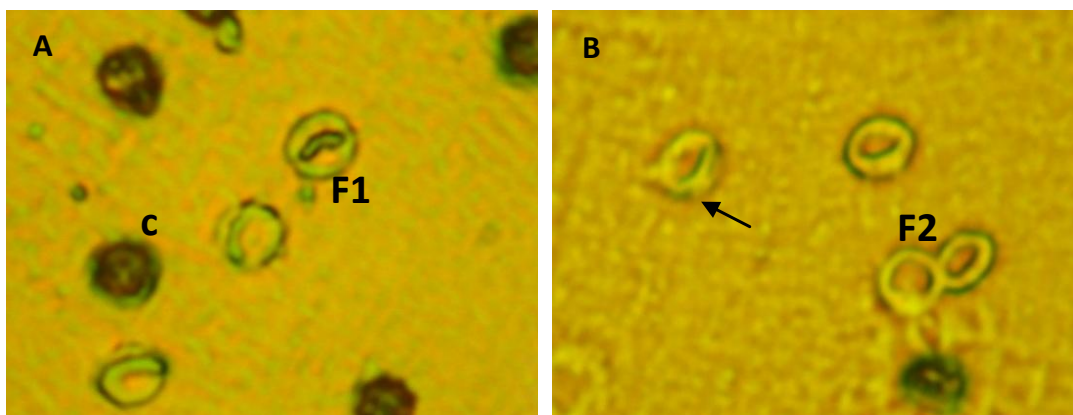


Figure 3. *Pf* infected erythrocytes shown under fluorescent filter in light microscopy (magnification $\times 1000$). C, Colloidal gold; F, labeled parasite.

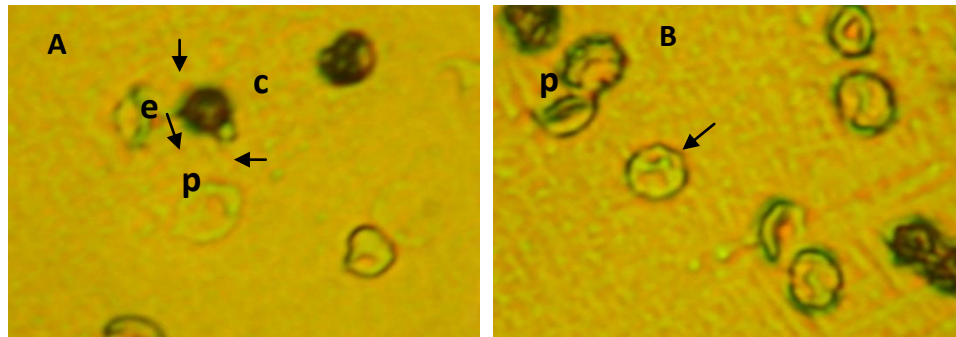


Figure 4. Demonstration of the specificity of *Pf*-HRP 2 to the antibody and the parasite in the infected erythrocyte (magnification $\times 1000$). c, Colloidal gold; p, parasite; e, erythrocyte.

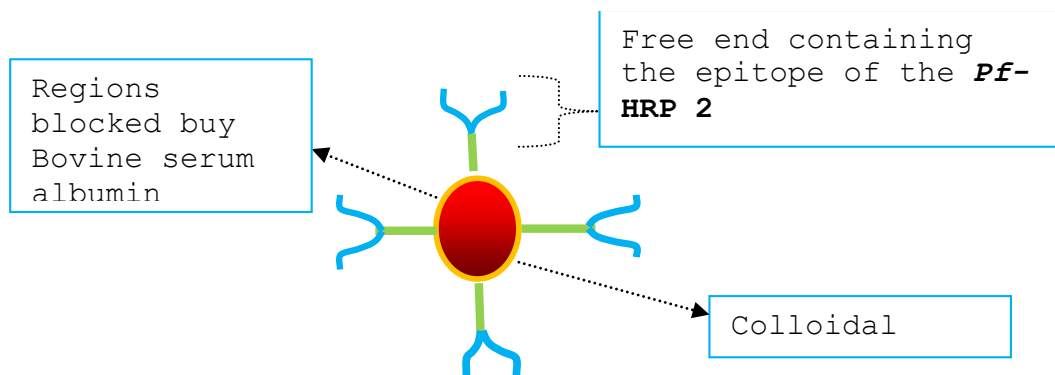


Figure 5. Schematic illustration of the multiplanar appearance of *Pf*-HRP 2 gold conjugate (Courtesy: Trinitron Biotech Limited, Nigeria).

and multiplication stage of the infection (magnification $\times 1000$). Arrow heads in Figure 4A shows the projecting free limbs of the antibodies, colloidal gold (c), parasite (p) and erythrocyte (e). Figure 4B shows parasite localized in infected erythrocytes at different stages of the infection (magnification $\times 1000$).

In living tissues, colloidal gold can be used to study molecular traffic and transport system. When labeled with appropriate antibodies, they localize in specific regions of the cell (Nolan et al., 2012). Thus, this technique was designed to localize the colloidal gold on the surface of erythrocytes and in the cytoplasm of cells housing the parasite in the blood and also free parasites in the blood. Considering the electrical properties of the surface of colloidal gold as being positively charged, and the localization of chains of negatively charged amino acids in the tail of the Y shaped antibodies (also *Pf*-HRP 2), an electrostatic force of attraction can occur between colloidal gold particles and the antibodies (Gao et al., 2012).

A major advantage of this technique is that it leaves the reactive part of the antibody unbounded such that they remain as free edges of the multiplanar gold conjugate

with two epitope per one Y shaped antibody and possessing four of such planar structures.

The planar structure as described in Figure 5 gives the colloidal gold conjugate stability and shows that aggregation has not occurred during the preparation of the gold conjugate. This technique has eliminated the false positive observed post treatment; when the specimen is incubated with the gold conjugate, it will show that the protein is present but the parasite is absent, thus breaking the tie for a false positive malaria test post treatment. It will also aid quick counting of parasite in the blood, as it tags both parasite and parasite infected cells, helping in diagnosis and treatment.

This feature is also of advantage over the RDTs, as the colour formation (chromoaggregation of colloidal gold conjugates) will most likely give a uniform coloration, from 750 parasites/ μl till 3000 parasites/ μl of blood. In this study, it is observed that the parasites are specifically labeled and can be counted on the slide to give a definitive count, by locating bounded conjugates. Since this technique utilizes antibody, it will be of advantage in identifying parasites microscopically; antibody-antigen

reactions are specific thus giving the advantage of specifically identifying plasmodium falciparum even at lower magnifications. This technique can be employed for a wide range of blood infections including determination of the extent of spread of cancer cells. It can be used to target infection sites for live studies in tissues and to treat cancers, as the conjugated colloidal gold can be irradiated to explode tropically within tumor cells thus destroying the cancers.

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