*Original Article*

# Usefulness of a new immunochromatographic assay using fluorescent silica nanoparticles for serodiagnosis of Thai patients with amebiasis

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**SUMMARY**     A fluorescence immunochromatography (FIC) kit was developed recently using fluorescent silica nanoparticles coated with a recombinant C-terminal fragment of the surface lectin intermediate subunit (C-Igl) of *Entamoeba histolytica* to establish rapid serodiagnosis of amebiasis. We further evaluated the system using serum samples from 52 Thai patients with amebiasis. Of the patients, 50 (96%) tested positive using FIC. The samples were also tested using enzyme-linked immunosorbent assay (ELISA) with C-Igl as the antigen. Two samples were negative on ELISA but positive on FIC. The correlation coefficient between the fluorescence intensity using FIC and the optical density value using ELISA was 0.5390, indicating a moderate correlation between the two tests. Serum samples from 20 patients with malaria and 22 patients with *Clostridioides difficile* infection were also tested using FIC. The false-positive rates were 4/20 (20%) and 1/22 (4%) in patients with malaria and *C. difficile* infection, respectively. Combining the data from the present study with our previous study, the sensitivity and specificity of FIC were determined to be 98.5% and 95.2%, respectively. The results of the 50 samples were studied using a fluorescence scope and a fluorescence intensity reader, and the findings were compared. Disagreements were found in only two samples showing near-borderline fluorescence intensity, indicating that the use of scope was adequate for judging the results. These results demonstrate that FIC is a simple and rapid test for the serodiagnosis of amebiasis.

*Keywords*     laboratory diagnosis, *Entamoeba histolytica*, lectin intermediate subunit, fluorescence immunochromatography

## 1. Introduction

Amebiasis, caused by the protozoan parasite *Entamoeba histolytica*, is a major disease in both developing and developed countries. Annually, an estimated 50 million cases of colitis and liver abscesses result in 55,000 deaths worldwide (*1*). Laboratory diagnosis of amebiasis by the detection of organisms, antigens, or DNA of *E. histolytica* is crucial for the early initiation of treatment (*2-4*). Serological tests to detect antibodies against *E. histolytica* are also useful, particularly in cases of extra-intestinal amebiasis. Several recombinant proteins of *E. histolytica* have been identified as candidates for the serodiagnosis of amebiasis (*5-9*).

We have previously demonstrated that the recombinant C-terminal fragment of the surface lectin intermediate subunit (C-Igl) of *E. histolytica* is useful as an antigen for enzyme-linked immunosorbent assays (ELISA) (*10-12*) as well as for multiple infectious disease detection systems (*13*). We recently developed a sensitive immunochromatographic kit using fluorescent silica nanoparticles coated with C-Igl for rapid serodiagnosis of amebiasis (*14*). However, all serum samples from the patients with amebiasis in the study were obtained from Japan.

Therefore, in the present study, we examined serum

samples from Thai patients with amebiasis to determine whether this kit is suitable for a wider application in Asia. Because several false-positive results were found in serum samples from patients with malaria and *Clostridioides difficile* infection in a previous study (*14*), further evaluation was performed using additional serum samples from patients with these infectious diseases. We also evaluated the use of a handheld fluorescence scope to facilitate the reading of the results.

## 2. Materials and Methods

### 2.1. Serum samples

Serum samples from a total of 52 patients with amebiasis in Thailand were obtained from the King Chulalongkorn Memorial Hospital. Of the patients, 30 samples were from patients with liver abscesses and 4 were from amebic dysentery. The patients were confirmed to respond to metronidazole treatment. The remaining 18 suspected cases were determined based on positive serology. All of the samples tested positive using an indirect hemagglutination (IHA) test (IHA Cellognost® Amoebiasis, Behring Diagnostics, Marburg, Germany) with various titers ranging from 1:128 (lowest positive) to ≥ 1:4,096 (highest positive). These samples were also confirmed to be positive using an indirect fluorescent antibody (IFA) test at a titer of 1:64. IFA was performed as described previously (*15,16*). Serum samples from 20 patients with malaria diagnosed in Thailand and serum samples from 22 patients with *C. difficile* infection obtained from Tokai University Hospital in Japan were used for the evaluation. Serum samples from 60 healthy Japanese individuals with negative serology in the IFA test were used as negative controls for ELISA to determine the cut-off value. The serum samples were stored at -80ºC, -30ºC or -20ºC before use. The study conformed to the provisions of the Declaration of Helsinki (as revised in 2013, *https://wma.net/what-we-do/medical-ethics/declaration-of-helsinki*). This study was approved by the review boards of Tokai University (17R017) and Chulalongkorn University (246/61).

### 2.2. Fluorescence immunochromatography

The immunochromatographic kit used in this study was prepared as described previously (*14*). Twenty µL of serum sample and 60 µL of 50 mM borate buffer (pH 8.0) were added to a tube containing freeze-dried silica nanoparticles. After rehydrating the particles, the solution was added to the well of an immunochromatographic cassette. After 30 min, the cassette was scanned using an immunochromatographic reader (DiaScan; Otsuka Electronics, Osaka, Japan), and the optimized value in the reader window was recorded. The measurement was repeated twice and the mean value was used. Based on a previous study, the cut-off fluorescence

intensity value was set to 2,181 (*14*). Cassettes showing various fluorescence intensities were selected using an immunochromatographic reader and the fluorescence intensity of each sample was measured using a handheld fluorescence microscope. Each cassette was set in a QD Scope (Furukawa Electric Advanced Engineering, Ichihara, Japan) and then judged as positive or negative by three raters independently, based on the recognition of an obvious fluorescent band with the naked eye. The fluorescence intensity was measured again using a DiaScan α reader, and the value was used for the analysis of correlation with judgment based on the fluorescence scope.

### 2.3. Enzyme-linked immunosorbent assay

ELISA using recombinant C-Igl as an antigen was performed as previously described (*11*). The wells of 96-well flat-bottom Costar EIA/RIA plates (Corning Incorporated, Kennebunk, ME) were incubated with 100 ng of C-Igl in 50 mM sodium bicarbonate buffer (pH 9.6) overnight at 4°C. The wells were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween) and then treated with PBS containing 1% skim-milk for 1 h. A total of 100 µL of serum diluted 1:400 with PBS was added to each well and incubated for 1 h at 23°C. After washing with PBS-Tween, 100 µL of horseradish peroxidase-conjugated goat immunoglobulin G (IgG) to human IgG (whole molecule; MP Biomedicals, Solon, Ohio) diluted 1:2,000 with PBS containing 1% skim-milk was added to each well and the wells were incubated for 1 h at 23°C. After being washed with PBS-Tween, the wells were incubated with 200 µL of substrate solution (0.4 mg/mL of *o*-phenylenediamine in citric acid-phosphate buffer [pH 5.0] containing 0.001% hydrogen peroxide). After 30 min, the reaction was stopped by the addition of 50 µL of 2 M $H_2SO_4$ and the optical density (OD) value at 490 nm was measured using a SpectraMax i3 plate reader (Molecular Devices Japan, Tokyo, Japan). The cut-off value for a positive result was defined as an OD value > 3 standard deviations above the mean of 60 healthy negative controls.

### 2.4. Statistical analysis

Descriptive statistics including frequency and percentage were calculated for characteristics of the samples. Mean and Standard divisions were calculated for the continuous variables. Sensitivity and Specificity for the tests were calculated using the data from the present study as well as the data from the previous study. The level of significance was fixed at $P = 0.05$ and any value less than or equal to 0.05 was considered to be statistically significant. Prism 6 (GraphPad Software, San Diego, Calif., USA) was used for plotting the values and for analysis.

## 3. Results

### 3.1. Evaluation of fluorescence immunochromatography using serum samples from Thai patients with amebiasis

The 52 serum samples from Thai patients with amebiasis were tested using FIC. The correlation between fluorescence intensity and the IHA titer is shown in Figure 1. Of the samples, 50 tested positive for FIC. One of the four samples with the lowest IHA-positive titer (1:128) and one of the five samples with an IHA titer of 1:256 were scored as negative on FIC. However, all of samples with IHA titers ≥ 1:512 were positive on FIC indicating that the sensitivity of FIC in Thai patients was 96.2% (95% CI, 86.8-99.5).

### 3.2. Comparison of fluorescence immunochromatography and the enzyme-linked immunosorbent assay

The 52 serum samples from Thai patients with amebiasis were also examined using ELISA with recombinant C-Igl as the antigen. The correlation between the fluorescence intensity on FIC and the OD value on ELISA is shown in Figure 2. Using an OD cut-off value of 0.201 based on the mean plus 3 standard deviations of samples from healthy Japanese population, two samples were scored as negative, demonstrating a sensitivity of 96.2% for ELISA. One of the two serum samples with negative results had the lowest positive IHA titer, and the other had an IHA titer of 1:1024; these were positive for FIC. The correlation coefficient between the fluorescence intensity on FIC and the OD value on ELISA was 0.5390, indicating a moderately positive correlation between the two tests.

### 3.3. Specificity of fluorescence immunochromatography

Because several false-positive reactions were recorded in patients with malaria and *C. difficile* infection in a previous study, further evaluation of serum samples from patients with these infectious diseases was performed in the present study. Four of 20 serum samples (20%) from the malaria patients and one of the 22 serum samples (4.5%) from patients with *C. difficile* infection were scored as positive for FIC (Figure 3). When the five samples were tested by ELISA and IFA, none were positive, indicating that the positive reaction to FIC was a false positive. In the evaluation of FIC, by the addition of data from the present study to our previous study, the sensitivity and specificity were determined to be 98.5% ([80 + 50]/[80 + 52]; 95% CI, 94.6-99.8) and 95.2% ([122 + 37]/[125 + 42]; 95% CI, 90.8-97.9), respectively.

### 3.4. Evaluation of reading of the fluorescence immuno-chromatography result using a hand-held viewer

Fifty cassettes showing a range of fluorescence intensity values were read using a QD Scope fluorescence viewer (Figures 4A and 4B). The correlation between the positive rates by the three raters using a QD Scope and the fluorescence intensity read by DiaScan is shown in Figure 4C. Among the serum samples showing a fluorescence intensity of less than 2,181, two samples with values of 2,072 and 2,071 were scored as positive by all three and two of the three raters, respectively. The
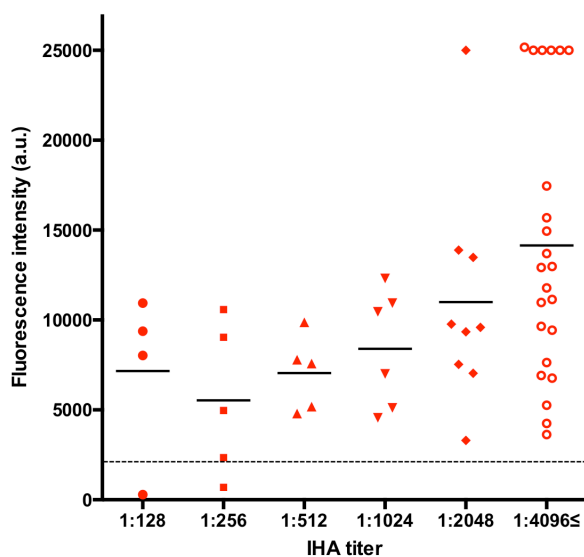


**Figure 1. Correlation between fluorescence intensity on fluorescence immunochromatography and the indirect hemagglutination titer of 52 serum samples from Thai patients with amebiasis.** The horizontal bars show the mean intensity. The broken line shows the cut-off value. For samples with fluorescence intensity above the measurement range using a DiaScan α reader, the value was recorded as 25,000.
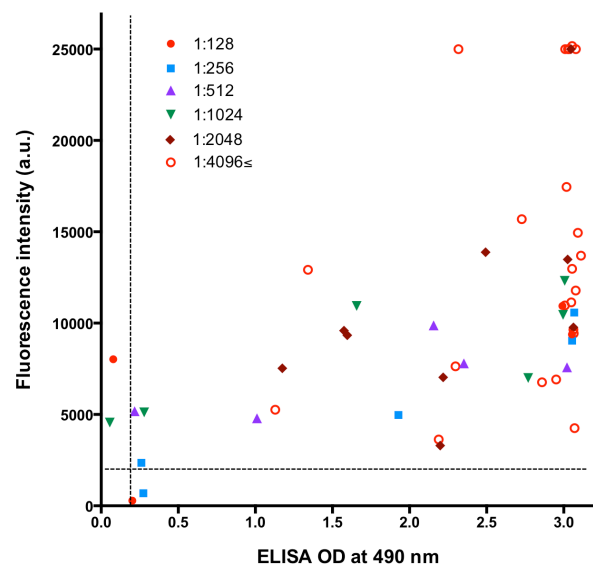
**Figure 2. Correlation between fluorescence intensity on fluorescence immunochromatography and the enzyme-linked immunosorbent assay optical density value of 52 serum samples from Thai patients with amebiasis.** The broken lines show the cut-off value. The indirect hemagglutination titers are shown by various types of dots.

results of the other samples were the same by the two reading methods.

## 4. Discussion

The present study demonstrated the high sensitivity of the FIC kit for testing serum samples from Thai patients with amebiasis. The use of C-Igl for the serodiagnosis of amebiasis by ELISA and FIC was originally studied using serum samples from patients with amebiasis collected in Japan (*11,14*). ELISA using C-IgL was useful for the epidemiological surveys of *E. histolytica*-infection in China (*10,12*). It has also been demonstrated

that C-Igl is a useful antigen for studying microfluidic devices in China (*17*). In an evaluation of a microsphere-based multiplex assay using C-Igl, the sensitivity and specificity were 100% using serum samples from patients with amebic liver abscesses from Bangladesh with serum samples from Japan as negative controls (*13*). These results suggest that C-Igl is a useful antigen for the serodiagnosis of amebiasis in all over Asia using several methods, including FIC. This may be because the primary structure of C-Igl is well-conserved among *E. histolytica* strains derived from various countries (Tachibana *et al.*, unpublished data).

In addition, this study demonstrated that the sensitivity of the FIC was comparable to that of ELISA using same samples. Recombinant C-Igl was used as the antigen in both tests, which confirmed the suitability of C-Igl despite the different detection systems used. ELISA is useful for testing large numbers of samples, whereas the FIC may be useful for testing small numbers of samples. It would be useful to have several different assays with different characteristics for the serodiagnosis of amebiasis. As false-positive reactions were recorded in two of nine samples from patients with malaria and in one of ten samples from patients with *C. difficile* infection in a previous study (*14*), further evaluation of serum samples from patients with these infectious diseases was performed in the present study. In this study, there was a relatively high rate of false-positive results in serum samples from patients with malaria on testing using FIC, whereas only one of the 22 serum samples from patients with *C. difficile* infection was false-positive. As these serum samples tested negative using ELISA with the same antigen, this rules out the possibility that the false-positivity rate on FIC was due to the existence of common epitopes
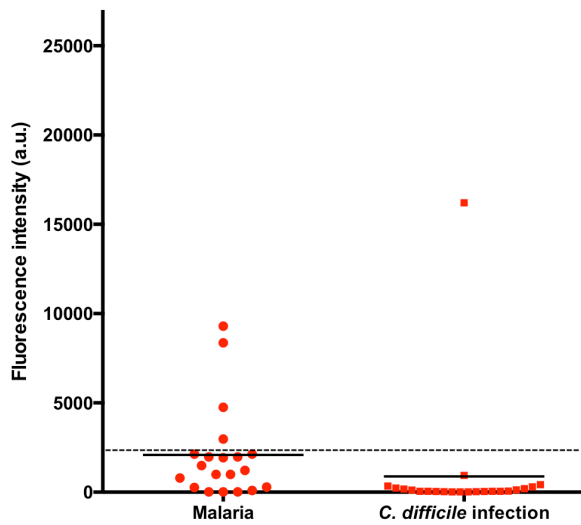


**Figure 3. Fluorescence intensity on fluorescence immuno-chromatography of serum samples from patients with malaria and *C. difficile* infection.** The horizontal bars show the mean intensity. The broken line shows the cut-off value.
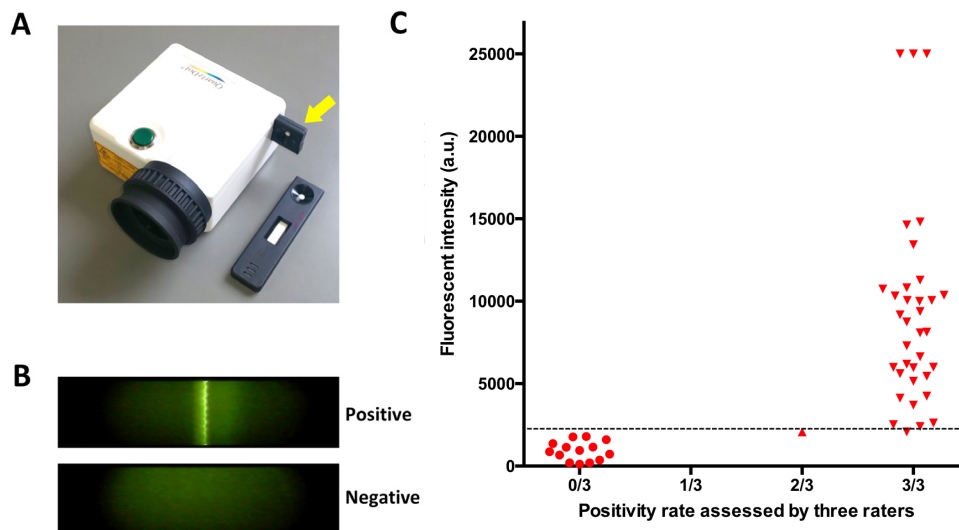


**Figure 4. Application of a QD Scope for assessment of positive serology on fluorescence immunochromatography (FIC).** (A) Photograph of the QD Scope and a cassette used in this study. The arrow indicates the inserted cassette. (B) Photograph of a fluorescent band in the window of the cassette. Representative pattern of positive and negative cases on FIC. (C) Correlation between the fluorescence intensity on immunochromatography and the positivity rate assessed by three raters using a QD Scope. The broken line shows the cut-off value on FIC.

between C-Igl and *Plasmodium* antigens. There is a chance that the sera of some malaria patients contain an unknown factor that affects the reaction to FIC. If such a factor exists, the factor can be removed during the washing step after the incubation of serum samples with C-Igl, using an ELISA system. The principle of this FIC system is that C-Igl on the surface of fluorescent silica nanoparticles and another C-Igl on the membrane are linked by bivalent IgG and/or divalent IgM molecules specific for C-Igl (*14*). Another possible system for capturing specific antibodies bound to C-Igl on the particles is the use of anti-human IgG and IgM antibodies on the membrane. It will be valuable to test the system in future studies because of the possibility that false positives will be reduced in addition to the detection of IgG and IgM separately (*18*).

In our previous study, the sensitivity and specificity of the FIC were 100 % and 97.6%, respectively (*14*); and by combining the present data, the values decreased to 98.5% and 95.2%, respectively. However, these values are comparable to the sensitivity and specificity of commercial ELISA and IHA kits of 69.0-100% and 87.5-99.8%, respectively (*2,19-22*).

Fluorescence assays have the advantage of higher intensity compared with visible wavelengths; however, specialized equipment is required to detect fluorescence (*18,23*). A handheld reader of fluorescence intensity, such as DiaScan, α is compact and useful for quantitative evaluation, but relatively expensive, and its program must be customized for the kit (*14*). In contrast, fluorescence viewers are economical and versatile without customization (*24*). This study showed that the results obtained using the two reading methods were consistent, except for samples with borderline values. Additionally, the fluorescence viewer exhibited excellent interrater reliability. This study demonstrated that the use of a handheld fluorescence scope was adequate for reading the results of the FIC assay.

In conclusion, the FIC kit using fluorescent silica nanoparticles and C-Igl appear to be potentially useful for the simple and rapid serodiagnosis of amebiasis in Asia. This study confirmed that the assay has high sensitivity and specificity except for a 20% false positive rate in patients with malaria. Additionally, the use of a fluorescence scope was effective in distinguishing between positive and negative results.

## Acknowledgements

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