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Development of a sensitive immunochromatographic kit using fluorescent silica nanoparticles for rapid serodiagnosis of amebiasis

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Research Article

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Abstract

We have previously shown that the C-terminal region of the intermediate subunit of *Entamoeba histolytica* galactose- and *N*-acetyl-D-galactosamine-inhibitable lectin (C-Igl) is a useful antigen for serodiagnosis of amebiasis. An immunochromatographic kit was developed using fluorescent silica nanoparticles coated with C-Igl prepared in *Escherichia coli*. Samples for examination were added to the freeze-dried particles and then applied to the immunochromatographic device, in which a test line on the membrane was also coated with C-Igl. Fluorescent intensity was measured using a hand-held reader. In an evaluation of the kit using a human monoclonal antibody, the minimum amount of C-Igl specific antibody showing positive results was 100 pg. In the evaluation of serum samples with different antibody titers in indirect immunofluorescent antibody tests in the kit, 20 μL of serum was sufficient to obtain positive results at 30 min. Serum samples from symptomatic patients with amebic colitis and amebic liver abscess and those from asymptomatic *E. histolytica*-cyst carriers showed positive results in the kit. Based on evaluation using sera from healthy controls and patients with other infectious diseases, the sensitivity and specificity of the kit were 100 and 97.6%, respectively. Therefore, we conclude that the newly developed kit is useful for rapid serodiagnosis of amebiasis.

Introduction

Amebiasis caused by infection with the enteric protozoan parasite *Entamoeba histolytica* is a major parasitic disease affecting people in both developing and developed countries. *Entamoeba histolytica* causes an estimated 50 million cases of dysentery, colitis and extraintestinal abscesses that result in 40 000–110 000 deaths annually (Stanley, 2003). Laboratory diagnosis of amebiasis is important to start treatment promptly. Intestinal amebiasis is usually diagnosed by detection of cysts or trophozoites. However, there is a need to discriminate between *E. histolytica* and morphologically identical nonpathogenic species *Entamoeba dispar*, *Entamoeba moshkovskii* and *Entamoeba bangladeshi* by polymerase chain reaction (PCR) analysis or detection of *E. histolytica*-specific antigens, especially in cases of asymptomatic cyst passers (Haque *et al.*, 2000; Heredia *et al.*, 2012; Royer *et al.*, 2012; Verkerke *et al.*, 2015). In addition, in extra-intestinal amebiasis, it is not easy to detect trophozoites in a liver abscess. Therefore, a serological test is practical as a sensitive and noninvasive means of diagnosis of the amebic liver abscess. Detection of serum antibody is also effective, even in asymptomatic cyst passers infected with *E. histolytica* (Tachibana *et al.*, 2000).

Serological tests based on recombinant proteins of *E. histolytica* have recently been reported (Stanley *et al.*, 1991; Lotter *et al.*, 1992; Zhang *et al.*, 1992; Shenai *et al.*, 1996; Lee *et al.*, 2000; Ning *et al.*, 2013; Saidin *et al.*, 2014). The heavy subunit of galactose- and *N*-acetyl-D-galactosamine-inhibitable lectin (Hgl), which is the key molecule in amebic adherence to host cells and subsequent pathogenesis, is a useful antigen. Hgl forms a heterodimer with a light subunit (Lgl) *via* disulfide bonds. The intermediate subunit of the lectin (Igl) is non-covalently associated with the Hgl/Lgl dimer at different lipid raft-like domain compartments and also contributes to adherence (Cheng *et al.*, 2001; Laughlin *et al.*, 2004; Welter

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et al., 2011). Recombinant Igl, and especially the C-terminal region (C-Igl), is a useful antigen for serodiagnosis (Tachibana *et al.*, 2004). An ELISA for C-Igl can be used to examine a large number of serum samples (Chen *et al.*, 2007; Yang *et al.*, 2012), and C-Igl is also applicable for simultaneous diagnosis of multi-infectious diseases (Fujii *et al.*, 2014).

A rapid and simple method for serodiagnosis is required in hospital laboratories, and an immunochromatographic kit is one of the most useful methods, even for parasitic diseases (Wang *et al.*, 2013; Saidin *et al.*, 2014; Barbosa Junior *et al.*, 2015; Janwan *et al.*, 2016). However, there is a limitation in the sensitivity of immunochromatography because colloidal gold particles or colored latex beads are used for detection by visibility. Recently, silica nanoparticles containing a fluorescent dye have been developed for antigen detection (Toriyama *et al.*, 2015). In this study, we used fluorescein silica nanoparticles to detect a specific antibody in serum samples from patients with amebiasis and developed a new immunochromatographic kit for rapid serodiagnosis.

Materials and methods

Serum samples

A total of 205 serum samples were examined. Most were obtained from hospitalized patients and outpatients at Tokai University Hospital, Tokyo Metropolitan Komagome Hospital and St. Luke's International Hospital in Tokyo, and were stored at −80 °C before use (Tachibana *et al.*, 2004). Sera from 28 patients with a liver abscess and sera from 35 patients with amebic colitis, but without a liver abscess, were used as symptomatic cases. The diagnoses of these patients were based on their clinical symptoms, positive serology in an indirect immunofluorescent antibody (IFA) or indirect hemagglutination (IHA) test, ultrasound or computed tomography examination (liver abscess), endoscopic or microscopic examination (colitis) and prompt response to treatment with metronidazole. Initial sera collected from the patients suspected of having amebiasis were used for the assay. Sera from 17 asymptomatic cyst passers infected with *E. histolytica*, but not with *E. dispar*, were also used. Identification of the species was performed by PCR (Tachibana *et al.*, 1991). In addition, sera from 45 patients with other protozoan diseases, including *Blastocystis hominis*-infection (7 cases), giardiasis (6 cases), malaria (9 cases), toxoplasmosis (7 cases), leishmaniasis (9 cases) and Chagas disease (7 cases), were examined. Sera from 10 patients with *Clostridium difficile* infection were also used for evaluation. Sera from 70 healthy Japanese subjects with no evidence of present infections were used as controls. All samples used in this study were anonymized.

Human immunoglobulin

Human monoclonal antibody XEhI-H2, which is specific for *E. histolytica* Igls, was prepared as previously described (Tachibana *et al.*, 2009) and used for evaluation. Human immunoglobulin purified from plasma of healthy subjects was used as a control (Tachibana *et al.*, 2009).

Preparation of the immunochromatographic kit

Recombinant C-Igl was prepared in *Escherichia coli* as previously described (Tachibana *et al.*, 2004). Fluorescent silica nanoparticles (Quartz Dot, Furukawa Electric Co. Ltd.) were synthesized by repeated polymerization of fluorescent dye and silica. Particles with a diameter of approximately 280 nm were used. Sulfhydryl groups on the surface of the particles were converted to amino groups, and C-Igl was bound to the amino groups *via* *N*-hydroxysuccinimide. Aliquots of the particles were dispensed into plastic tubes and then freeze-died. C-Igl was also sprayed onto a nitrocellulose membrane to form a test line using an XYZ3060 Dispense Platform (BioDot, Irvine, CA). The membrane was treated with a blocking reagent and then dried at 37 °C for 30 min and then at 23 °C overnight. The membrane, sample pad and absorbent pad were assembled on a laminated membrane card. The assembled sheet was cut into 5-mm strips and then placed into a plastic cassette with windows.

Immunochromatographic assay

A total of 80 μL of serum sample and 50 mM borate buffer (pH 8.0) were added to a tube containing freeze-dried silica nanoparticles. After rehydration of the particles, the solution was added to the well of an immunochromatographic cassette. After incubation at 23 °C, the cassette was set in a hand-held immunochromatographic reader (DiaScan α, Otsuka Electronics, Osaka, Japan). The membrane in the cassette was scanned at 891 spots at 20-μm intervals to measure fluorescent intensity. The optimized value was estimated automatically by the reader, and the value displayed in the reader window was recorded. The measurement was repeated twice and an average value was used. Fluorescence intensity data were sent *via* Bluetooth wireless connection to a personal computer and analysed by DiaScan Utility software, v.1.0 (Otsuka Electronics). Detection of peak fluorescent intensity and comparisons were performed on Microsoft Excel 2010.

Statistics

GraphPad Prism v.6.0 (San Diego, CA) was used for statistical analysis and graphing.

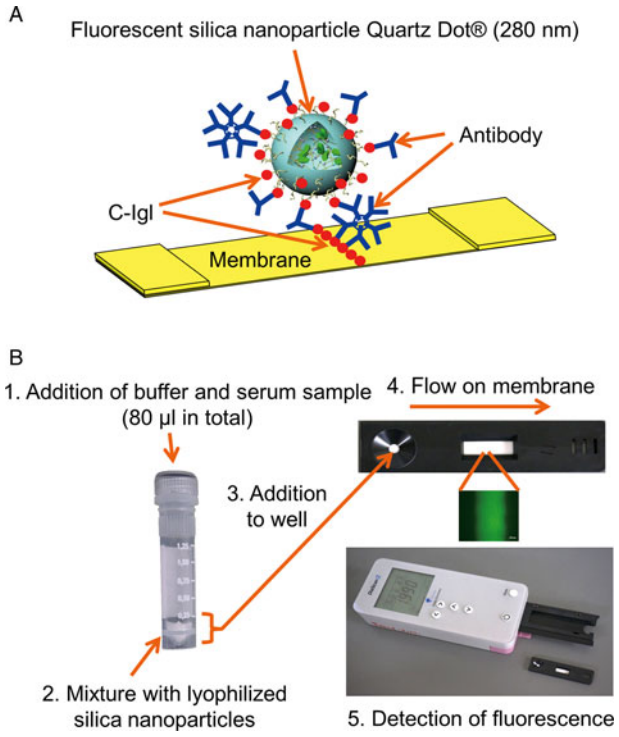

Fig. 1. Schematic chart of immunochromatographic assay kit using fluorescent silica nanoparticles coated with *E. histolytica* C-Igl for serodiagnosis of amebiasis. (A) Principle of detection of antibodies to C-Igl. If specific antibodies to C-Igl are present, fluorescein silica nanoparticles are captured on the membrane *via* the antibodies. (B) Procedure of the assay. Numbers 1–5 show each step in the procedure. In step 5, a cassette in the assay kit is set in a tray of hand-held reader DiaScan α (Otsuka Electronics) and then scanned. Intensity is displayed digitally on a window of the reader and scanning data is sent to a personal computer *via* a Bluetooth wireless connection. The positive fluorescent image was taken using a fluorescein microscope.

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Results

Preparation of the immunochromatographic assay kit

The principles of the detection system for antibodies to C-Igl in the immunochromatographic assay are shown in Fig. 1A. Recombinant C-Igl prepared in *Escherichia coli* was bound on the surface of silica nanoparticles and also pre-immobilized on a nitrocellulose membrane to form a test line. By evaluation using a human monoclonal antibody, XEhI-H2, specific to Igl, the concentration of C-Igl was optimized to yield distinct fluorescent intensity in comparison with a control with no antibody in the system. The established procedure for the assay kit developed in this study is shown in Fig. 1B. Fluorescence intensity was measured using a hand-held reader.

Detection limit

To determine the minimum amount of antibody that can be detected using the kit, various amounts of XEhI-H2 were applied using the kit. As shown in Fig. 2A, the fluorescent intensity increased in a dose- and time-dependent manners. With 100 ng of XEhI-H2, the intensity saturated at 15 min after incubation. Positive intensity was still observed for 100 pg of XEhI-H2. In controls, there was no difference in fluorescent intensity between 1 μg and 10 ng of normal human antibodies. Scanning waveforms of fluorescence from various antibody concentrations at 30 min after incubation are shown in Fig. 2B. The waveform peak was detected for 100 pg of XEhI-H2, while the waveform for the control antibody was flat at 1 μg. Therefore, the minimum amount of antibody detectable by the kit was judged to be 100 pg.

Influence of the serum volume and incubation time

Three serum samples from patients with amebiasis with different antibody titers in an IFA were used to determine the volume of serum required for the kit (Fig. 3). A negative serum in the IFA test was used as a control. For serum with a high IFA titer of 1:1024, strong fluorescence was observed even at 5 μL of the sample (Fig. 3A). To yield an apparent intensity level for serum with a low IFA titer of 1:64, incubation for 30 min with 20 μL of serum or 15 min with 40 μL of serum was adequate (Fig. 3C and D). The condition of 20 μL of serum and incubation for 30 min was finally selected. Scanning waveforms of fluorescence in immunochromatography were confirmed using serum samples from two patients and two healthy controls. Apparent peaks corresponding to the test line were observed for patients with amebic colitis and asymptomatic cyst passers, whereas waveforms in healthy controls were flat (Fig. 4).

Evaluation using sera from patients with amebiasis and other infectious diseases

To evaluate the fluorescent immunochromatographic kit, sera from 35 patients with amebic colitis, 28 patients with amebic liver abscess and 17 asymptomatic cyst passers were measured (Fig. 5). Sera from 70 healthy Japanese subjects with no evidence of infectious disease were used as negative controls. Receiver

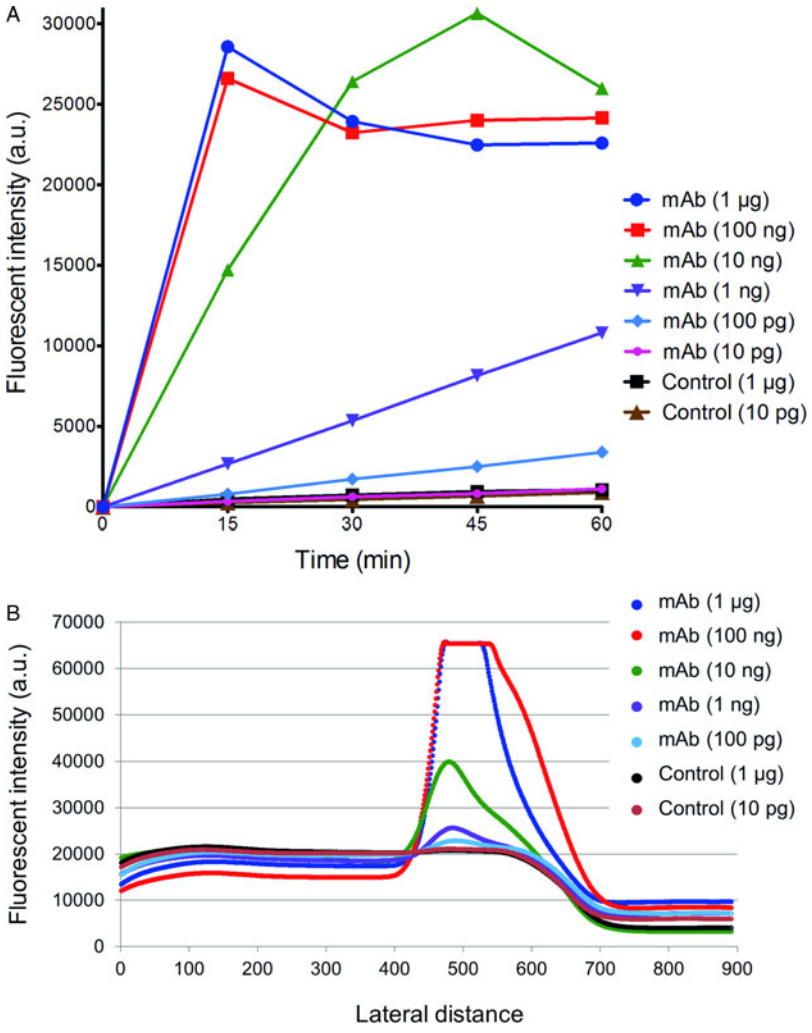

Fig. 2. Immunochromatography using a human monoclonal antibody specific to C-Igl. (A) Reactivity of various amounts of human monoclonal antibody XEhI-H2 in the kit. Fluorescein intensity was measured at 15, 30, 45 and 60 min after addition to the well. (B) Scanning waveforms of fluorescence in immunochromatography at 30 min.

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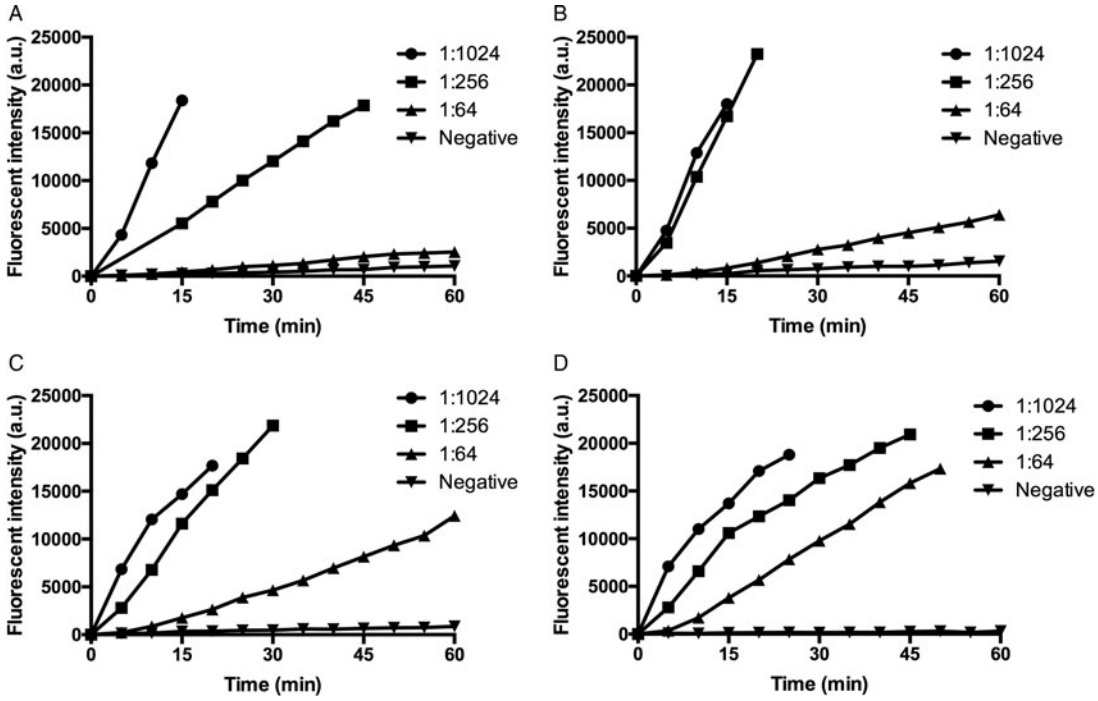

Fig. 3. Fluorescent intensities from serum samples in the immunochromatographic assay. The serum volumes were 5 μL (A), 10 μL (B), 20 μL (C) and 40 μL (D). Three positive sera with different IFA titers and an IFA-negative serum were used. Fluorescein intensity was measured at 5 min intervals after addition to the well.

operating characteristic (ROC) curve analysis showed AUC, 1.000; 95% CI, 1.000 to 1.000 (Fig. 6). The cut-off value was determined to be 2,181. To examine cross-reactivity with other pathogens, 55 sera from patients with giardiasis, *Blastocystis hominis* infection, toxoplasmosis, malaria, Chagas disease, leishmaniasis and *Clostridium difficile* infection were also evaluated. Two samples from patients with malaria and one from a patient with *C. difficile* infection were scored as positives (Fig. 5). Based on the analysis of sera from amebiasis and that from healthy controls and other infectious diseases, the sensitivity and specificity of the kit were calculated as 100% and 97.6%, respectively. Since the turnaround time of the immunochromatographic kit was about 30 min, the kit was shown to be useful for rapid serodiagnosis of amebiasis.

Discussion

In this study, a new immunochromatographic kit for detection of serum antibody to *E. histolytica* antigen was developed using fluorescent silica nanoparticles. Quartz Dot is a silica nanoparticle that contains a high concentration of organic dye with high brightness, which makes it possible to lower the detection limit of the kit. Indeed, 100 pg of specific antibody to C-Igl could be detected in the kit with use of monoclonal antibody XEhI-H2. However, since antibodies to C-Igl in real serum samples are polyclonal and recognize several different epitopes of the protein, it is difficult to evaluate the exact detection limit. Recently, it has been reported that an immunochromatographic assay kit using fluorescent silica nanoparticles bound with a monoclonal antibody is useful for detection of *Acanthamoeba* antigen, even in clinical samples that are negative in direct smear or culture tests (Hiwatashi *et al.*, 1997; Toriyama *et al.*, 2015). Five trophozoites or 40 cysts in samples have been detected using the assay kit. Another advantage of a fluorescence kit is its more quantitative nature in comparison with an immunochromatographic kit with colloidal gold or latex particles. In the present study, a new handheld reader was used instead of a fluorescent scope (Toriyama *et al.*, 2015) and was useful to show the fluorescent intensity of a test line. In addition, diminishing of fluorescent intensity on the membrane over time was negligible, and was <20% after 7 months at room temperature (unpublished data).

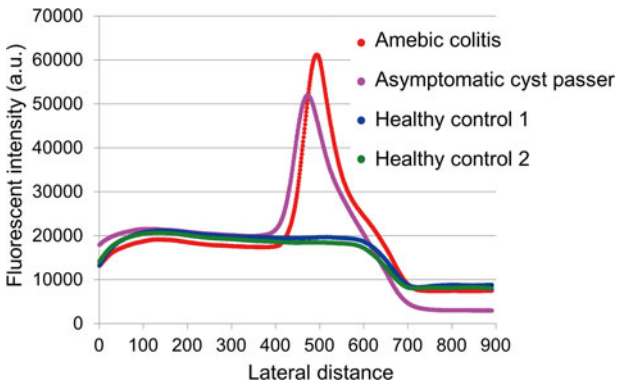

Fig. 4. Evaluation of scanning waveforms of fluorescence in immunochromatography using human sera. Sera (20 μL) from a patient with amebic colitis, an asymptomatic cyst passer, and two healthy Japanese controls were used. Fluorescent intensity was measured at 15 min after addition to the well.

We have previously shown that C-Igl is a useful antigen for serodiagnosis of amebiasis in an ELISA (Tachibana *et al.*, 2004; Chen *et al.*, 2007; Yang *et al.*, 2012). C-Igl has higher sensitivity and specificity than other fragments of Igl and has been applied in serodiagnostic systems using a microfluidic device and a microsphere-based multiplex assay (Zhao *et al.*, 2013; Fujii *et al.*, 2014). In the present study, all serum samples from patients with amebiasis, including asymptomatic cyst passers, were judged to be positive in our kit, showing that its sensitivity was 100%. However, two sera from patients with malaria and one from a patient with *C. difficile* infection were also scored as positive. To exclude the possibility that these three patients were coinfected with *E. histolytica*, the reactivity of these samples to C-Igl was analysed using our previously reported ELISA system (Tachibana *et al.*, 2004). None of the samples showed positive

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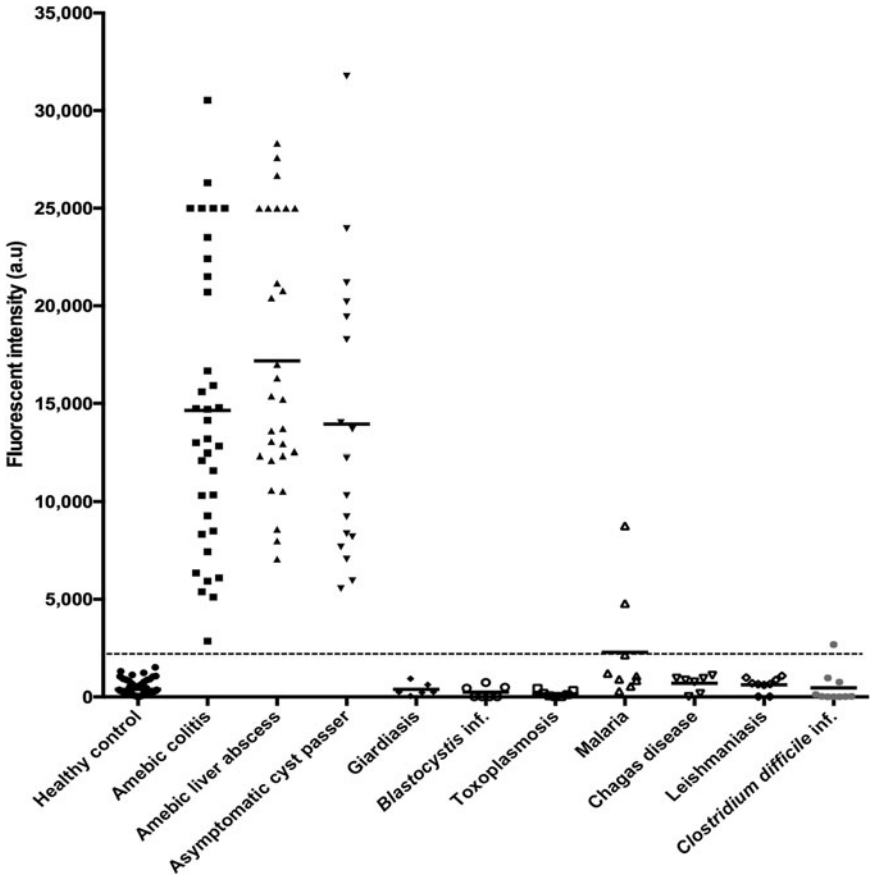

Fig. 5. Fluorescent intensity of sera from healthy controls and patients with amebiasis and other infectious diseases in the immunochromatographic assay. Horizontal bars show the mean intensity. The broken line shows the cut-off value. For samples with fluorescent intensity in DiaScan α above the measurement range, the value was recorded as 25 000.

results and therefore, the reason for these false-positives is unclear. If other infectious disease groups are included in controls in ROC curve analysis (AUC, 0.9981; 95% CI, 0.9944–1.002), the cut-off value becomes 4944 with 98.75% sensitivity and 99.2% specificity. Further evaluation is required using a large number of serum samples, including those from patients with malaria, *C. difficile* infection and *E. dispar* infection in addition to healthy controls from endemic areas, to establish an appropriate cut-off.

C-Igl is a candidate vaccine for prevention of amebic liver abscess formation (Min *et al.*, 2016). Immunization with C-Igl induces humoral immunity with increased expression of IL-4 and IL-10, and it has also been shown that C-Igl possesses both hemolytic and cytotoxic activities (Kato *et al.*, 2015, 2017). A human monoclonal antibody recognizing C-Igl can inhibit hemolytic activity *in vitro* and amebic liver abscess formation of hamsters by passive immunization (Tachibana *et al.*, 2009; Kato *et al.*, 2015). The observation that all sera from asymptomatic cyst passers were scored as positive in this study may support the finding that antibodies to C-Igl function protect against invasive amebiasis.

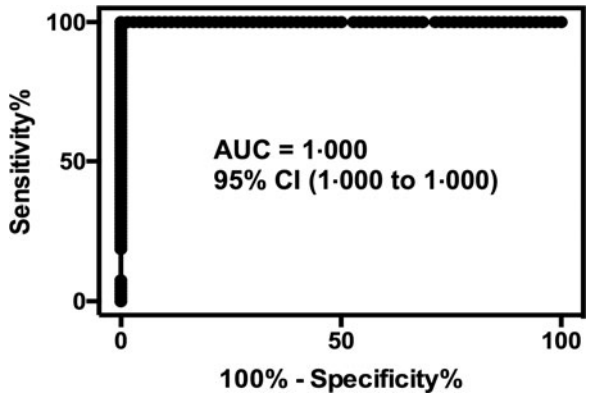

Fig. 6. Fitted ROC curves from the *E. histolytica*-infected and healthy control groups.

There are several commercial ELISA and IHA kits for serodiagnosis of amebiasis. ELISA is widely thought to be adequate for clinical purposes (Tanyuksel and Petri, 2003) and an ELISA system is useful for examination of a large number of serum samples simultaneously but is less convenient for a single sample. IHA is easy to perform, but its lower sensitivity may lead to false-negative results in comparison with ELISA (del Carmen Sanchez-Guillen *et al.*, 2000). IHA is also a quantitative assay, but the serial dilution of samples is required. The method reported here does not require the dilution steps of serum samples required in both ELISA and IHA. The sensitivity and specificity of commercial ELISA or IHA kits have been reported as 72.2–100% and 90.9–99.1%, respectively (Tanyuksel and Petri, 2003; Flores *et al.*, 2016), and the sensitivity and specificity of this study are comparable with these values. In addition, the short turnaround time of the immunochromatographic kit is an advantage in comparison with ELISA and IHA.

In conclusion, an immunochromatographic kit using fluorescent silica nanoparticles with C-Igl as antigen was shown to be useful for rapid serodiagnosis of amebiasis. To our knowledge, this is the first report of the development of an antibody detection system using fluorescent silica nanoparticles.

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Declaration of interest. T. Kamada and H. Watanabe are employees of Furukawa Electric Advanced Engineering Co., Ltd. The other authors declare no conflicts of interest.

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Ethical standards. The study was approved by the Review Board of Tokai University (17R017).

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