Development of an Immunochromatographic Assay Kit Using Fluorescent Silica Nanoparticles for Rapid Diagnosis of Acanthamoeba Keratitis

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We developed an immunochromatographic assay kit that uses fluorescent silica nanoparticles bound to anti-Acanthamoeba antibodies (fluorescent immunochromatographic assay [FICGA]) and evaluated its efficacy for the detection of Acanthamoeba and diagnosis of Acanthamoeba keratitis (AK). The sensitivity of the FICGA kit was evaluated using samples of Acanthamoeba trophozoites and cysts diluted to various concentrations. A conventional immunochromatographic assay kit with latex labels (LICGA) was also evaluated to determine its sensitivity in detecting Acanthamoeba trophozoites. To check for cross-reactivity, the FICGA was performed by using samples of other common causative pathogens of infectious keratitis, such as Pseudomonas aeruginosa, Staphylococcus aureus, and Candida albicans. Corneal scrapings from patients with suspected AK were tested with the FICGA kit to detect the presence of Acanthamoeba, and the results were compared with those of real-time PCR. The FICGA kit detected organisms at concentrations as low as 5 trophozoites or 40 cysts per sample. There were no cross-reactivities with other pathogens. The FICGA was approximately 20 times more sensitive than the LICGA for the detection of Acanthamoeba trophozoites. The FICGA kit yielded positive results for all 10 patients, which corresponded well with the real-time PCR results. The FICGA kit demonstrated high sensitivity for the detection of Acanthamoeba and may be useful for the diagnosis of AK.

Acanthamoeba keratitis (AK) is a severe and sight-threatening ocular infection which usually occurs in the context of soft contact lens wear or trauma. It is caused by Acanthamoeba spp., which inhabit various environments, such as lakes, oceans, soil, and tap water (1–3). Acanthamoeba can assume two different morphological forms: the trophozoite, which can utilize nutrition and proliferate, and the dormant protective cyst, which can withstand high temperatures, desiccation, and pharmacologic insults. Acanthamoeba can transform between the trophozoite and cyst forms to adjust to various environments (3–6). The incidence of AK has increased dramatically in recent years, a trend which has been attributed to the increasing prevalence of soft contact lens wear and usage of contact lens disinfectant solutions that do not prevent the growth of Acanthamoeba (7, 8). Since the clinical manifestations of AK are similar to those of herpes simplex keratitis, the condition can often be misdiagnosed (9–11). Therefore, reliable detection of Acanthamoeba is essential for an accurate diagnosis of AK. As delayed diagnosis has been associated with poor visual outcomes (12, 13), it is important to identify a method for the rapid and specific diagnosis of AK.

Microscopic examination and culture of corneal scrapings are the diagnostic procedures conventionally used to detect Acanthamoeba (14). Microscopic examination of corneal smears stained with Fungiflora Y, calceinfluor white stain, and acridine orange stain has been reported to be an effective method of diagnosing AK (15–17), but these tests require technical expertise, and a false negative can occur if there is an insufficient sample from the corneal scraping. Culturing live Acanthamoeba isolates is time-consuming, and a long incubation time is needed to confirm Acanthamoeba growth. This results in decreased sensitivity of the test and delays in starting treatment (14, 18).

Recently, highly sensitive PCR procedures which amplify Acanthamoeba DNA have been used in the diagnosis of AK (19–22). Real-time PCR can also provide quantitative values for Acanthamoeba DNA copy numbers, enabling clinicians to estimate the efficacy of AK treatment (23, 24). However, these genetic procedures require expensive specialized equipment and technical expertise. Moreover, these tests are available only in certain facilities, such as academic centers.

Immunochromatographic assays (ICGA) are useful for antigen detection, and they can generally be completed within 30 min and do not require specialized equipment or expertise (25–29). Because of its rapidity and simplicity, the ICGA is utilized in many clinical tests, such as pregnancy tests and tests which detect antigens from causative pathogens. We developed an ICGA that uses fluorescent silica nanoparticles bound to anti-Acanthamoeba antibodies and evaluated its efficacy for the detection of Acanthamoeba and the diagnosis of Acanthamoeba keratitis (AK). The sensitivity of the ICGA kit was evaluated using samples of Acanthamoeba trophozoites and cysts diluted to various concentrations. A conventional ICGA kit with latex labels (LICGA) was also evaluated to determine its sensitivity in detecting Acanthamoeba trophozoites. To check for cross-reactivity, the FICGA was performed by using samples of other common causative pathogens of infectious keratitis, such as Pseudomonas aeruginosa, Staphylococcus aureus, and Candida albicans. Corneal scrapings from patients with suspected AK were tested with the FICGA kit to detect the presence of Acanthamoeba, and the results were compared with those of real-time PCR. The FICGA kit detected organisms at concentrations as low as 5 trophozoites or 40 cysts per sample. There were no cross-reactivities with other pathogens. The FICGA was approximately 20 times more sensitive than the LICGA for the detection of Acanthamoeba trophozoites. The FICGA kit yielded positive results for all 10 patients, which corresponded well with the real-time PCR results. The FICGA kit demonstrated high sensitivity for the detection of Acanthamoeba and may be useful for the diagnosis of AK.
pathogens, such as viruses and bacteria (25–27). In the field of ophthalmology, it is used for the diagnosis of adenoviral conjunctivitis and herpetic keratitis (28, 29). Colloidal gold and latex, each of which serves as a label when coupled to an antibody, are used to visualize antigens in ICGA kits for adenovirus and herpesvirus, respectively (28, 29). ICGA kits that use colloidal gold or latex labels usually show results using a detection line which appears on the membrane in either blue or red. However, checking for this detection line with only the naked eye is not ideal, as it may reduce the test’s sensitivity or lead to false positives (30).

In this study, an ICGA kit using an anti-Acanthamoeba antibody was developed to detect Acanthamoeba. A fluorescent substance, instead of colloidal gold or latex, was used to label the anti-Acanthamoeba antibody, and the detection line was visualized using a portable fluorescence microscope. The ICGA kit used in this study (the fluorescent immunochromatographic assay [FICGA]) consists of a test strip, extraction liquid containing surfactant, and fluorescent silica nanoparticles (Quartz Dot; Furukawa Electric Co., Ltd.), each coupled with an antibody for Acanthamoeba castellanii. Quartz Dot is an amorphous silica nanoparticle with a diameter of approximately 290 nm and is harmless to the human body. Since the surface of the particle is covered by highly hydrophilic hydroxyl groups, the particles disperse uniformly in the sample solution or buffer. Nonspecific adsorption due to hydrophobic interactions is infrequent, thereby minimizing fluorescent noise and maximizing sensitivity. Moreover, the antibody-fluorescent label complex is stabilized by strong covalent modifications on its surface and has high luminescence since it contains a high concentration of the fluorochrome rhodamine 6G. Rhodamine 6G is an ideal fluorescent marker because it fluoresces at 555 nm, a wavelength to which the eye is extremely sensitive under light-adapted conditions (31). We used mouse monoclonal antibodies to Acanthamoeba castellanii, which were produced as previously described (32). These antibodies recognize pathogenic Acanthamoeba spp. but not any other amoebas (32). The aim of this study was to investigate the efficacy of a FICGA for the detection of Acanthamoeba and diagnosis of AK.

MATERIALS AND METHODS

Immunochromatographic assay for detection of Acanthamoeba. To perform the assay, a sample was treated with 200 μl of extraction liquid and freeze-dried fluorescent silica nanoparticles, and 80 μl of this mixture was placed on the edge of a test strip which had been previously sprayed with anti-Acanthamoeba antibodies. Thirty minutes after applying the sample mixture, the fluorescent emission was observed with a portable fluorescence microscope (Immuno Chromato-Reader; Furukawa Electric Co., Ltd.) (Fig. 1). The fluorescent intensity of the test line was also measured with a specialized fluorescent scanner 60 min after application of the sample. The fluorescence microscope can detect signals at an intensity of approximately 100 arbitrary units.

A second ICGA kit was developed with the same mouse-derived anti-Acanthamoeba antibodies labeled with latex markers (the latex-labeled immunochromatographic assay [LICGA]). For this kit, equal parts of sample fluid and antibody solution were mixed, and 100 μl of this mixture was applied to a test strip with an anti-Acanthamoeba antibody test line and a mouse IgG control antibody line. The test result was confirmed within 30 min after sample application. A red band at both the test and control antibody line positions was considered a positive result, whereas a negative result consisted of a red band at the control position only.

In vitro examination. Acanthamoeba castellanii strain ATCC 30011 was purchased from the American Type Culture Collection and used to examine the sensitivity of the FICGA and LICGA kits. Trophozoites were grown axenically in peptone-yeast-extract-glucose (PYG) medium at 25°C in a tissue culture flask (Becton Dickinson, Tokyo,

FIG 1 Principle of the fluorescent immunochromatographic assay kit. The sample is treated with extraction liquid and mixed with anti-Acanthamoeba monoclonal antibodies, which are conjugated to fluorescent silica nanoparticles. Acanthamoeba antigens, if present in the sample, form complexes with antibodies which are fixed in place on a strip. Fluorescent emission is observed by using a portable fluorescence microscope. If there are no Acanthamoeba antigens in the sample, no fluorescent band is observed.
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Immunochromatographic Assay for Acanthamoeba

TABLE 1 Detection of Acanthamoeba by fluorescent immunochromatographic assay

| Morphological form | Result at an Acanthamoeba concentration (no. of organisms) of:
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Trophozoites</td>
<td>-</td>
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<tr>
<td>Cysts</td>
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*+, positive; -, negative; NT, not tested.

RESULTS

In vitro examination. The aim of this study was to evaluate the sensitivity of the LICGA and the FICGA for the detection of Acanthamoeba. The thresholds for detecting Acanthamoeba trophozoites with the LICGA are shown in Fig. 2. Three independent assays showed similar results, namely, that trophozoites were detected via this method at concentrations of >100 trophozoites per sample. The thresholds for detecting Acanthamoeba trophozoites and cysts with the FICGA kit are shown in Table 1. The FICGA kit detected trophozoites at concentrations as low as 5 organisms per sample and cysts at concentrations as low as 40 organisms per sample. The FICGA was approximately 20 times more sensitive than the LICGA for detecting Acanthamoeba trophozoites. There was a significant positive correlation between the intensities of the fluorescent test lines (as measured by a specialized fluorescent scanner) and the Acanthamoeba concentrations in the trophozoite and cyst forms (Fig. 3).

When samples of P. aeruginosa, S. aureus, and C. albicans were used, the FICGA yielded negative results, and there were no cross-reactivities.

Clinical evaluation. Samples from all 10 patients tested positive for Acanthamoeba DNA when evaluated by real-time PCR (Table 2). The maximum copy number was $4.0 \times 10^5$ copies per sample, and the minimum was <25 copies per sample. The
FICGA kit detected *Acanthamoeba* in all 10 patients, consistent with the results of real-time PCR. In 7 patients who were evaluated by smear analysis and culture, the smears and cultures were both positive in 2 patients. Either the culture or the smear was positive in 2 patients, and both studies were negative in the remaining 3 patients. All patients responded well to treatment with antiamoebic therapy with topical biguanides and antifungals.

**DISCUSSION**

In recent years, amplification of *Acanthamoeba* DNA by PCR has become the principal procedure for detecting *Acanthamoeba* and enabling sensitive diagnosis of AK (19–24). However, PCR has some limitations, as previously stated. A rapid and readily available procedure would help to ensure an accurate diagnosis at the first visit and reduce the risk of inappropriate treatments that complicate the clinical picture and make diagnosis more difficult. In the current study, we succeeded in developing an ICGA kit for improving the accuracy of pathogen detection in a variety of infectious diseases. Although a specialized fluorescence microscope is required to read the test strips, it is a small device which is easy to acquire and is relatively inexpensive, and therefore it can be made available in nearly any local clinic.

In conclusion, we developed a novel ICGA kit using fluorescent silica nanoparticles for the rapid diagnosis of AK. Our in vitro studies suggest that this kit is highly sensitive for the detection of *Acanthamoeba castellanii*, and when the test was applied to clinical specimens, the FICGA results corresponded closely with the results of real-time PCR. Although further evaluation of this technique is needed, the FICGA kit seems to be useful for the diagnosis of AK as it is more rapid and simpler to use than conventional diagnostic procedures, including PCR.

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