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Bioconjugated fluorescent silica nanoparticles for the rapid detection of *Entamoeba histolytica*

Ahmad Hemadi[a], Alireza Ekrami[b], Hormozd Oormazdi[a], Ahmad Reza Meamar[a], Lame Akhlaghi[a], Ali Reza Samarbaf-Zadeh[c], Elham Razmjou[a,*]

[a] Department of Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[b] Infectious and Tropical Diseases Research Center and Nanotechnology Research Centre, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[c] Department of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

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ABSTRACT

Rapid detection of *Entamoeba histolytica* based on fluorescent silica nanoparticle (FSNP) indirect immunofluorescence microscopy was evaluated. Silica nanoparticles were synthesized using Stöber's method, with their surface activated to covalently bind to, and immobilize, protein A. For biolabeling, FSNP was added to conjugated *E. histolytica* trophozoites with monoclonal anti-*E. histolytica* IgG1 for microscopic observation of fluorescence. Fluorescent silica nanoparticle sensitivity was determined with axenically cultured *E. histolytica* serially diluted to seven concentrations. Specificity was evaluated using other intestinal protozoa. Fluorescent silica nanoparticles detected *E. histolytica* at the lowest tested concentration with no cross-reaction with *Entamoeba dispar*, *Entamoeba moshkovskii*, *Blastocystis* sp., or *Giardia lamblia*. Visualization of *E. histolytica* trophozoites with anti-*E. histolytica* antibody labeled with fluorescein isothiocyanate (FITC) was compared with that using anti-*E. histolytica* antibody bioconjugated FSNP. Although FITC and FSNP produced similar results, the amount of specific antibody required for FITC to induce fluorescence of similar intensity was fivefold that for FSNP. Fluorescent silica nanoparticles delivered a rapid, simple, cost-effective, and highly sensitive and specific method of detecting *E. histolytica*. Further study is needed before introducing FSNP for laboratory diagnosis of amoebiasis.

1. Introduction

Entamoeba histolytica is a protozoan parasite that causes amoebiasis, infecting approximately 50 million people worldwide with 40,000 to 100,000 deaths annually (Stanley, 2003; van Hal et al., 2007). Early and accurate diagnosis could reduce transmission and mortality of amoebiasis. Light microscopy examination is the most common, rapid, and simple method of identifying *E. histolytica*, although it has low sensitivity and specificity because of difficulty in distinguishing *E. histolytica* from two morphologically identical species, *Entamoeba dispar* and *Entamoeba moshkovskii*, and requires an expert reader to differentiate it from other species of *Entamoeba* and even macrophages (Ngui et al., 2012; Tanyuksel and Petri, 2003). Molecular diagnoses are the most accurate, but are relatively time-consuming and require expensive equipment and specialized personnel (Fotedar et al., 2007, 2008; Tanyuksel and Petri, 2003). Several rapid tests based on antigen detection that can differentiate between pathogenic and nonpathogenic *Entamoeba* using monoclonal antibodies have been introduced for *E. histolytica* identification in fecal samples (Fotedar et al., 2007; Haque et al., 2000). Fluorescein isothiocyanate (FITC) conjugated specific antibody has been shown to be simple, effective, and sensitive for the detection of several microorganisms (Majumdar et al., 2011).

The use of nanoparticles, especially silica nanoparticles, is a novel approach for detection of micro-organisms (Rossi et al., 2006; Salata, 2004). The Stöber method produces good quality nanoparticles of appropriate size in large quantities (Stöber et al., 1968). Despite the use of fluorescent silica nanoparticles for identification of pathogenic bacteria (Ekrami et al., 2011; Qin et al., 2007), their utilization in detection of *E. histolytica* has not been investigated. This study evaluated the use of bio-conjugated fluorescent silica nanoparticles as a simple, rapid, and sensitive method for detection of *E. histolytica* trophozoites. The study also, for the first time, compared FITC-labeled specific monoclonal antibody of *E. histolytica* for microscopic identification of this protozoan to results obtained with anti-*E. histolytica* antibody bioconjugated FSNP.

* Corresponding author at: Department of Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences, P.O. Box 15875-6171, Tehran, Iran. Tel.: +98 21 88622653; fax: +98 21 88622653
E-mail address: razmjou.e@iums.ac.ir (E. Razmjou).

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2. Materials and methods

2.1. Synthesis and bio-conjugation of fluorescent silica nanoparticles (FSNP)

To synthesize nanoparticles, Triton X-100 (1.77 mL), cyclohexan (7.5 mL), n-hexanol (1.8 mL), and 0.48 mL Tris (2,2′-bipyridyl) dichlororuthenium (II) hexahydrate solution (Rubpy) (20 mM) were combined and thoroughly mixed. To continue the reaction, 100 μL tetraethyl orthosilicate (TEOS) was added and placed in a mixer at 25 °C for 20 min. To initiate the process of polymerization, 60 mL of ammonia solution (NH_4OH) was added and incubated 24 h at 25 °C. Acetone was added 1:1 and vortexed for 5 min to complete the polymerization. To harvest nanoparticles, the solution was centrifuged at 3000 × *g* and washed three times in 95% ethanol. To avoid agglomeration of nanoparticles, following each washing process the sediment was sonicated (60 mA) for 30 s. After the third washing, the precipitate was air-dried in a Petri dish in dark. After complete drying, the orange powder of FSNP was collected in a test tube.

An atomic force microscope (SPM probe scanner DS 95–50 E, Danish microengineering A/S, Herlev, Denmark) and particle size analyzer (Analysette 22-Nano Tec Fritsch, Germany) were used to visualize the morphology and to confirm the size of the silica nanoparticles of approximately 60 nm.

The surface of the FSNPs was activated with cyanogen bromide to covalently bind to protein A of *Staphylococcus aureus* (Langone, 1982). A sample of 11.2 mg of nanoparticles was dissolved in 2 mL of sodium carbonate solution (2 M) by sonication (60 mA for 30 s). Then, 2 mL of a solution of 0.78 g cyanogen bromide in 2 mL acetonitrile was gently added over the course of 5 min, while rotating at 25 °C. For removing unbound chemical compounds, cold dH_2O was added to the solution and washed twice at 4500 × *g* for 8 min, followed by washing twice with 0.01 M PBS (pH 7.4).

The molecule of protein A was covalently bound to activated nanoparticles by adding 25 μL protein A aqueous solution (1 mg/mL) to 1 mL of FSNP solution and mixing for 24 h at 4 °C. To avoid non-specific binding, 6 mL of 3 M glycine (pH 8) was added at 4 °C and mixed completely for 16 h. The solution was then washed with PBS and held at 4 °C until use, this compound was stable at least for 6 months.

The protein A conjugated to FSNP was measured by the Bradford method (Bradford, 1976), and the presence of the amide group was confirmed using an infrared spectrophotometer (Bomem MB 155 S FTIR spectrometer, ABB Bomem, Inc., Canada). The emission and absorbance of nanoparticles were evaluated by a spectrofluorometer (RF-5301 PC, Shimadzu, Japan) and an ultraviolet–visible spectrophotometer (Varian cary-100, Australia).

2.2. Entamoeba sample preparation

Entamoeba histolytica reference strains HM-1, IMSS Clone 6 (ATCC® Number, 50527 ™), HK-9 (ATCC® Number, 30015™, Z-2), and *E. moshkovskii* (ATCC® Number, 30042™) were used. Trophozoites of *E. histolytica* strains and *E. moshkovskii* were axenically cultivated in TYS medium at 35.5 °C, as described (Clark and Diamond, 2002). *Entamoeba dispar* (Sahebani et al., 2005) was cultivated in xenic culture. To achieve *E. histolytica* concentrations of 5, 10, 20, 40, 80, 160, and 320/mL, a specific volume of homogenized culture media was serially diluted, following the initial amoeba count.

2.3. Biolabeling of Entamoeba histolytica with FSNP

For conjugation of a specific monoclonal antibody to *E. histolytica*, 500 μL of amoeba culture (TYS Media) was washed with PBS at 1500 × *g* for 5 min and suspended in an equal volume of PBS. Subsequently, 5 μL of mouse monoclonal anti-*Entamoeba histolytica* IgG1 ([BD1535]–ab20956; ABCAM, UK) was employed against a specific lectin antigen of *E. histolytica* and incubated for 1 h at 37 °C.

For biolabeling of amoebae, 25 μL of FSNP was added to 500 μL conjugated *Entamoeba* and incubated for 1 h at 37 °C. Unbound FSNP was removed by washing twice with PBS at 1500 × *g* for 2 min. The biolabeled *Entamoeba* was observed and recorded using a fluorescent microscope (Olympus BX51 and DP70 digital camera system, Olympus America, Inc., Center Valley, PA) with a 450–490 nm band pass excitation and a 515 nm long pass emission filter.

2.4. DNA extraction and PCR assay

For DNA extraction, 500 μL of each of the seven concentrations of amoeba culture were washed with PBS at 1500 × g for 5 min and suspended in 200 μL of PBS. DNA of amoeba trophozoites was extracted using the QIAamp DNA Mini kit (Qiagen, Germany) according to manufacturer's directions. The PCR of *E. histolytica* was performed using EntaF and EntaR primers as described by Hamzah et al. (2006). The PCR reaction was designed to amplify a 166 bp fragment of a small-subunit rRNA gene of *E. histolytica* (Hamzah et al., 2006).

2.5. Fluorescein isothiocyanate conjugation

Fluorescein isothiocyanate (FITC) solution was prepared using EasyLink FITC conjugation kit (ab102885; ABCAM, UK) following manufacturer's procedure. For conjugation of FITC, 10 μL of EasyLink modifier reagent was added to 100 μL of mouse monoclonal anti-*E histolytica* IgG1 and mixed. The antibody sample was pipetted into a vial of FITC solution and held in dark for 3 h at 25 °C; 10 μL of quencher was added and gently mixed. The sample was ready for use after 30 min. For identification of *E. histolytica*, 100 μL of the FITC conjugation was added to 500 μL of *Entamoeba* culture and incubated at 37 °C for 1 h. The fluorescent amoebae were microscopically observed.

2.6. Sensitivity and specificity

The sensitivity of FSNP was determined using serial dilution of *Entamoeba* from 5 to 320/mL and compared with standard PCR at the same concentrations. To increase accuracy, each diluted sample was divided into portions for FSNP and PCR assays. The specificity of FSNP was evaluated using intestinal protozoa including *E. dispar*, *E. moshkovskii*, *Blastocystis* sp., and *Giardia lamblia*. All observations were conducted in duplicate.

3. Results

3.1. Synthesis and bioconjugation of FSNP and biolabeling of E. histolytica

The intended morphology and size of synthesized silica nanoparticles were confirmed by an atomic force microscope and particle size analyzer, respectively. Activation of the nanoparticle surface was shown by the presence of amide bonds on the surface using infrared spectrophotometer.

The biolabeled amoebae were observed using a fluorescent microscope on quartz microscope slides (UQG Optics, Cambridge, UK) for removing the fluorescence background (Fig. 1).