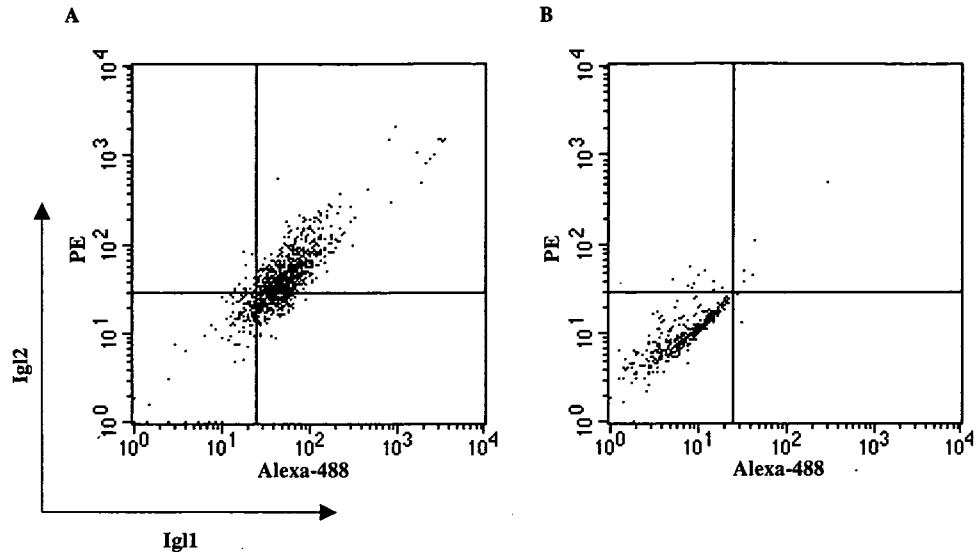

Fig. 5. Flow cytometric analysis of phenotypic expression of Igl1 and Igl2 on the surface of *Entamoeba dispar* trophozoites. Intact trophozoites from the SAW1734RclAR strain were double stained with monoclonal antibodies ED1-13 (specific for Igl1, IgG2b) and ED2-1 (specific for Igl2, IgG1), followed by Alexa Fluor 488-labelled goat anti-mouse IgG2b-specific antibody and PE-labelled goat anti-mouse IgG1-specific antibody (A). The control was stained only with secondary antibodies (B). The results are representative of 3 independent experiments.

trophozoites expressed both Igl1 and Igl2 on the cell surface.

Localization of Igls on trophozoites

Localization of Igl1 and Igl2 on *E. dispar* trophozoites was examined by confocal laser scanning microscopy using specific mAbs. Both Igls were localized on the plasma membrane and in cytoplasm in all trophozoites (Figs 6 and 7). However, the amount of each Igl in the trophozoites was variable, especially in the cytoplasm; that is, Igl1- and Igl2-dominant cells were present (arrows and arrowheads in Fig. 6). In addition, Igl1- and Igl2-dominant vacuoles were also observed within a single trophozoite (arrow and arrowhead in Fig. 7).

DISCUSSION

Comparison of the 2 *Igl* genes cloned from *E. dispar* with those from *E. histolytica* indicated differences in the sequences of the corresponding proteins in *E. dispar* and *E. histolytica* and in the 2 strains of *E. dispar*. However, all the cysteine residues, including the CXXC motifs, were conserved between species and between strains, which suggests that the fold of the protein is maintained and is important for its function. Amino acid identities of Igl1 and Igl2 within species (79% in *E. dispar* and 81% in *E. histolytica*; Cheng *et al.* 2001) were higher than those for each Igl between the 2 species (75–76% for Igl1 and 73–74% for Igl2). Insertions of 6 amino acids around position 840 in *E. dispar* were present in both Igl1 and Igl2, as shown in Fig. 1. Nucleotide identities of the 2 *Igl* genes were also higher within species, compared to the respective identities of the *Igl* genes between *E. dispar* and *E. histolytica*. These results suggest that duplication of the genes may have occurred after divergence of the species.

The properties of the Gal/GalNAc lectin of *E. histolytica* were demonstrated in a 150 kDa fraction purified by affinity chromatography using the *E. histolytica*-specific mAb EH3015 (Cheng *et al.* 1998). In addition, Igl1 and Igl2 of *E. histolytica* have been detected, in addition to Hgl and Lgl, in the protein fraction that binds specifically to GalNAc-BSA-coated magnetic beads (McCoy and Mann, 2005). However, when we performed a preliminary examination of the reactivity of recombinant Igls from *E. histolytica* and *E. dispar* with $GalNAc_{27}$-BSA by dot blot analysis and surface plasmon resonance, we could not prove that the recombinant proteins had sugar-binding properties (data not shown). Therefore, the Igls may exist as part of the lectin complex, perhaps with non-covalent association to another protein containing a sugar-binding site, with this association occurring either directly or being mediated by a third protein. Recently, it has been reported that Igl of *E. histolytica* is found in the protein fraction that interacts with purified brush border from human enterocytes (Seigneur *et al.* 2005). It has also been demonstrated that the 140 kDa fibronectin-binding molecule of *E. histolytica* (Talamas-Rohana *et al.* 1992) is identical with Igl2 (Hernandez-Ramirez *et al.* 2007). These observations indicate that Igls are important proteins for amoebic adherence to host cells, and since Igls are also expressed in the non-pathogenic amoeba, it seems likely that these proteins are important for colonization of amoebae in the large intestine.

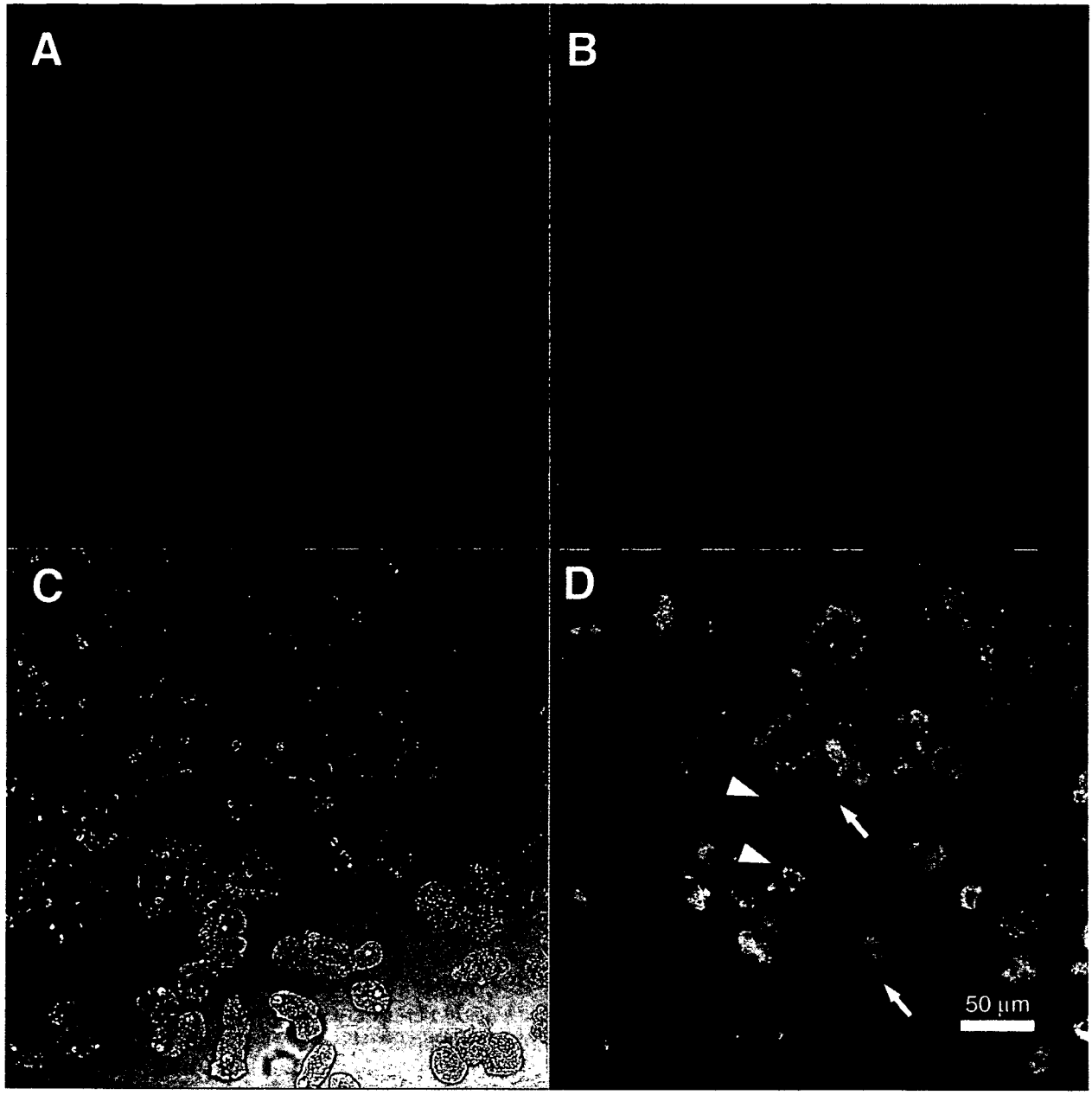

Fig. 6. Localization of Igl1 and Igl2 on trophozoites of *Entamoeba dispar* SAW1734RclAR observed by confocal laser scanning microscopy. Fixed trophozoites were stained with ED1-13 specific for Igl1 and a secondary Alexa Fluor 488-labelled anti-mouse IgG2b antibody (green) (A) or ED2-1 specific for Igl2 and a secondary Alexa Fluor 594-labelled anti-mouse IgG1 antibody (red) (B). Differential interference contrast microscopy is shown in (C). A merged image is shown in (D). Arrows and arrowheads indicate Igl1- and Igl2-dominant cells, respectively.

One of the interesting observations in this study is the difference in expression between Igl1 and Igl2. Since higher expression of Igl1 was observed at both protein and mRNA levels, the difference between the isoforms seems to be regulated mostly at the transcriptional level. The expression level of *Igl1* was also lower in *E. dispar* than in *E. histolytica*, whereas that of *Igl2* was comparable in the two species. It has been demonstrated that expression of *Hgl* (*Hgl2*) and *Lgl* (*Lgl1*) in *E. dispar* is lower than in *E. histolytica* (Pillai *et al.* 1997, 2001); therefore, Igl1 may be more closely associated with Hgl and Lgl. However, it is unknown whether the 2 Igl isoforms are associated with different isoforms of Hgl or Lgl. DNA microarray analyses have shown that a large number of genes are expressed differently in *E. histolytica* and *E. dispar*, and that there is a difference in gene expression between strains of *E. histolytica* of high and low virulence (Shah *et al.* 2005; MacFarlane and Singh, 2006; Davis *et al.* 2007). Lower expression of *Hgl* genes in *E. dispar* compared to *E. histolytica* has also been confirmed (MacFarlane and Singh, 2006), but *Lgl3* expression was found to be higher in an *E. histolytica* strain of low virulence compared to a strain of high virulence (Davis *et al.* 2007). *Igl* genes are down-regulated by

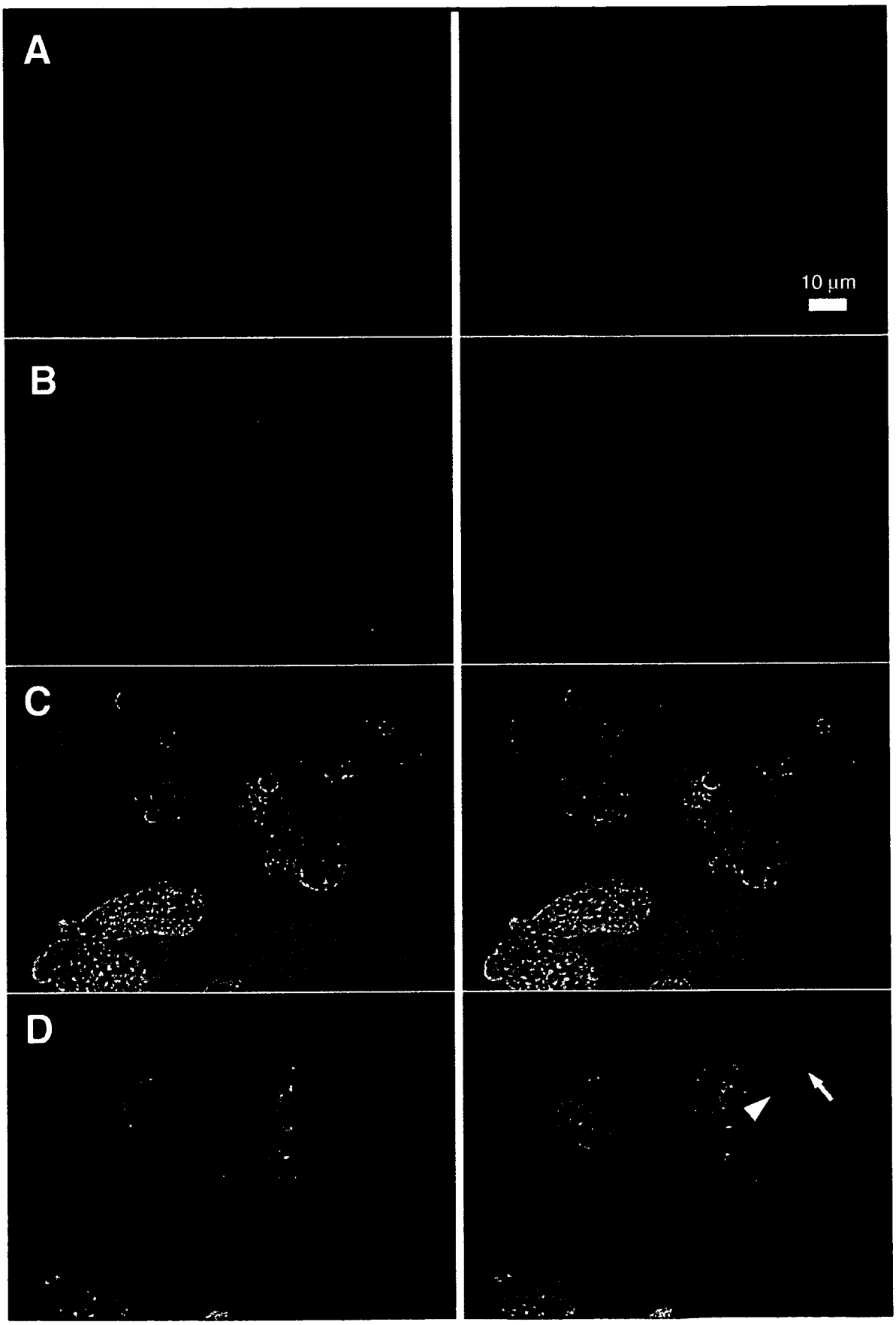

Fig. 7. Stereo images of localization of Igl1 (A) and Igl2 (B) on *Entamoeba dispar* trophozoites observed by confocal laser scanning microscopy. Fixed trophozoites were stained as described in Fig. 6. Differential interference contrast microscopy is shown in (C). A merged image is shown in (D). Arrow and arrowhead indicate single localization of Igl1 and Igl2, respectively.

heat shock stress, as are most *Hgl* and *Lgl* genes (Weber *et al.* 2006). Collectively, these observations suggest that the lectin as a whole is important for adherence and subsequent pathogenesis.

We also compared phenotypic expression of the two Igls in each trophozoite using flow cytometry and confocal microscopy. Interestingly, some trophozoites expressed Igl1 or Igl2 dominantly, although most cells expressed the two Igl proteins to a comparable extent, suggesting that both Igls are important for the amoeba. Igls were also localized in intracellular vacuoles of *E. dispar*. Recently, it has been demonstrated that Igl is contained in phagosomes of *E. histolytica* and that the quantity of Igl varies during phagosome maturation (Okada *et al.* 2005, 2006). The different localization of Igl1 and Igl2 in vacuoles suggests that the functions of the two Igls may differ or that their expression may vary during maturation of phagosomes or depending on certain cellular conditions. However, there may also be differences in the involvement of microtubules and proteases in phagosome maturation and degradation in *E. histolytica* and *E. dispar* (Mitra *et al.* 2005). In *E. histolytica*, different subcellular localization of the two Igl isoforms has yet to be shown.

In conclusion, this is the first study of the differences between Igl1 and Igl2 of *E. dispar*. Igl seems to be a vaccine candidate for amebiasis and may also be a useful antigenic molecule for specific serodiagnosis of amoebiasis (Cheng and Tachibana, 2001; Tachibana *et al.* 2004). Therefore, further studies of Igl are required to clarify its role in the host-parasite relationship.

This work was supported by a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science, and a grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare of Japan. X.-J. C. is a recipient of a Postdoctoral Fellowship for Foreign Researchers from the Japanese Society for the Promotion of Science.

REFERENCES

Bruchhaus, I., Jacobs, T., Leippe, M. and Tannich, E. (1996). *Entamoeba histolytica* and *Entamoeba dispar*: differences in numbers and expression of cysteine proteinase genes. *Molecular Microbiology* **22**, 255–263.

Cheng, X.-J., Hughes, M. A., Huston, C. D., Loftus, B., Gilchrist, C. A., Lockhart, L. A., Ghosh, S., Miller-Sims, V., Mann, B. J., Petri, W. A., Jr. and Tachibana, H. (2001). Intermediate subunit of the Gal/GalNAc lectin of *Entamoeba histolytica* is a member of a gene family containing multiple CXXC sequence motifs. *Infection and Immunity* **69**, 5892–5898.

Cheng, X.-J., Kaneda, Y. and Tachibana, H. (1997). A monoclonal antibody against the 150-kDa surface antigen of *Entamoeba histolytica* inhibits adherence and cytotoxicity to mammalian cells. *Medical Science Research* **25**, 159–161.

Cheng, X.-J. and Tachibana, H. (2001). Protection of hamsters from amebic liver abscess formation by immunization with the 150- and 170-kDa surface antigens of *Entamoeba histolytica*. *Parasitology Research* **87**, 126–130.

Cheng, X.-J., Tachibana, H. and Kaneda, Y. (1999). Protection of hamsters from amebic liver abscess formation by a monoclonal antibody to a 150-kDa surface lectin of *Entamoeba histolytica*. *Parasitology Research* **85**, 78–80.

Cheng, X.-J., Tsukamoto, H., Kaneda, Y. and Tachibana, H. (1998). Identification of the 150-kDa surface antigen of *Entamoeba histolytica* as a galactose- and *N*-acetyl-D-galactosamine-inhibitable lectin. *Parasitology Research* **84**, 632–639.

Davis, P. H., Schulze, J. and Stanley, S. L., Jr. (2007). Transcriptomic comparison of two *Entamoeba histolytica* strains with defined virulence phenotypes identifies new virulence factor candidates and key differences in the expression patterns of cysteine proteases, lectin light chains, and calmodulin. *Molecular and Biochemical Parasitology* **151**, 118–128.

Diamond, L. S. and Clark, C. G. (1993). A redescription of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt, 1925. *Journal of Eukaryotic Microbiology* **40**, 340–344.

Diamond, L. S., Harlow, D. R. and Cunnick, C. C. (1978). A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **72**, 431–432.

Hernández-Ramírez, V. I., Rios, A., Angel, A., Magos, M. A., Pérez-Castillo, L., Rosales-Encina, J. L., Castillo-Henkel, E. and Talamás-Rohana, P. (2007). Subcellular distribution of the *Entamoeba histolytica* 140 kDa FN-binding molecule during host-parasite interaction. *Parasitology* **134**, 169–177.

Khalifa, S. A., Imai, E., Kobayashi, S., Haghighi, A., Hayakawa, E. and Takeuchi, T. (2006). Growth-promoting effect on iron-sulfur proteins on axenic cultures of *Entamoeba dispar*. *Parasite* **13**, 51–58.

Kobayashi, S., Imai, E., Haghighi, A., Khalifa, S. A., Tachibana, H. and Takeuchi, T. (2005). Axenic cultivation of *Entamoeba dispar* in newly designed yeast extract-iron-gluconic acid-dihydroxyacetone-serum medium. *Journal of Parasitology* **91**, 1–4.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

MacFarlane, R. C. and Singh, U. (2006). Identification of differentially expressed genes in virulent and nonvirulent *Entamoeba* species: potential implications for amebic pathogenesis. *Infection and Immunity* **74**, 340–351.

McCoy, J. J. and Mann, B. J. (2005). Proteomic analysis of Gal/GalNAc lectin-associated proteins in *Entamoeba histolytica*. *Experimental Parasitology* **110**, 220–225.

Mitra, B. N., Yasuda, T., Kobayashi, S., Saito-Nakano, Y. and Nozaki, T. (2005). Differences in morphology of phagosomes and kinetics of acidification and degradation in phagosomes between the pathogenic *Entamoeba histolytica* and the non-pathogenic *Entamoeba dispar*. *Cell Motility and the Cytoskeleton* **62**, 84–99.

Okada, M., Huston, C. D., Mann, B. J., Petri, W. A., Jr. Kita, K. and Nozaki, T. (2005). Proteomic analysis of phagocytosis in the enteric protozoan parasite *Entamoeba histolytica*. *Eukaryotic Cell* **4**, 827–831.

Okada, M., Huston, C. D., Oue, M., Mann, B. J., Petri, W. A., Jr. Kita, K. and Nozaki, T. (2006). Kinetics and strain variation of phagosome proteins of *Entamoeba histolytica* by proteomic analysis. *Molecular and Biochemical Parasitology* **145**, 171–183.

Petri, W. A., Jr. Chapman, M. D., Snodgrass, T., Mann, B. J., Broman, J. and Ravdin, J. I. (1989). Subunit structure of the galactose and *N*-acetyl-D-galactosamine-inhibitable adherence lectin of *Entamoeba histolytica*. *Journal of Biological Chemistry* **264**, 3007–3012.

Petri, W. A., Jr. Haque, R. and Mann, B. J. (2002). The bittersweet interface of parasite and host: lectin-carbohydrate interactions during human invasion by the parasite *Entamoeba histolytica*. *Annual Review of Microbiology* **56**, 39–64.

Pillai, D. R., Britten, D., Ackers, J. P., Ravdin, J. I. and Kain, K. C. (1997). A gene homologous to hgl2 of *Entamoeba histolytica* is present and expressed in *Entamoeba dispar*. *Molecular and Biochemical Parasitology* **87**, 101–105.

Pillai, D. R., Kobayashi, S. and Kain, K. C. (2001). *Entamoeba dispar*: molecular characterization of the galactose/*N*-acetyl-D-galactosamine lectin. *Experimental Parasitology* **99**, 226–234.

Seigneur, M., Mounier, J., Prevost, M. C. and Guillen, N. (2005). A lysine- and glutamic acid-rich protein, KERP1, from *Entamoeba histolytica* binds to human enterocytes. *Cellular Microbiology* **7**, 569–579.

Shah, P. H., MacFarlane, R. C., Bhattacharya, D., Matese, J. C., Demeter, J., Stroup, S. E. and Singh, U. (2005). Comparative genomic hybridizations of *Entamoeba* strains reveal unique genetic fingerprints that correlate with virulence. *Eukaryotic Cell* **4**, 504–515.

Tachibana, H., Cheng, X.-J., Masuda, G., Horiki, N. and Takeuchi, T. (2004). Evaluation of recombinant fragments of *Entamoeba histolytica* Gal/GalNAc lectin intermediate subunit for serodiagnosis of amebiasis. *Journal of Clinical Microbiology* **42**, 1069–1074.

Tachibana, H., Ihara, S., Kobayashi, S., Kaneda, Y., Takeuchi, T. and Watanabe, Y. (1991). Differences in genomic DNA sequences between pathogenic and nonpathogenic isolates of *Entamoeba histolytica* identified by polymerase chain reaction. *Journal of Clinical Microbiology* **29**, 2234–2239.

Tachibana, H., Kobayashi, S., Cheng, X.-J. and Hiwatashi, E. (1997). Differentiation of *Entamoeba histolytica* from *E. dispar* facilitated by monoclonal antibodies against a 150-kDa surface antigen. *Parasitology Research* **83**, 435–439.

Talamás-Rohana, P., Rosales-Encina, J. L., Gutierrez, M. C. and Hernández, V. I. (1992). Identification and partial purification of an *Entamoeba histolytica* membrane protein that binds fibronectin. *Archives of Medical Research* **23**, 119–123.

Walsh, J. A. (1986). Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. *Reviews of Infectious Diseases* **8**, 228–238.

Weber, C., Guigon, G., Bouchier, C., Frangeul, L., Moreira, S., Sismeiro, O., Gouyette, C., Mirelman, D., Coppee, J. Y. and Guillen, N. (2006). Stress by heat shock induces massive down regulation of genes and allows differential allelic expression of the Gal/GalNAc lectin in *Entamoeba histolytica*. *Eukaryotic Cell* **5**, 871–875.

Willhoeft, U., Hamann, L. and Tannich, E. (1999). A DNA sequence corresponding to the gene encoding cysteine proteinase 5 in *Entamoeba histolytica* is present and positionally conserved but highly degenerated in *Entamoeba dispar*. *Infection and Immunity* **67**, 5925–5929.

Am. J. Trop. Med. Hyg., 77(5), 2007, pp. 825–828

Short Report: Seroprevalence of *Entamoeba histolytica* Infection in HIV-Infected Patients in China

Yi Chen, Yunzhi Zhang, Bin Yang, Tangkai Qi, Hongzhou Lu, Xunjia Cheng,* and Hiroshi Tachibana

Department of Medical Microbiology and Parasitology, Fudan University School of Medicine, Shanghai, China; Department of Infectious Diseases, Huashan Hospital Affiliated to Fudan University, Shanghai, China; Department of Infectious Diseases, Tokai University School of Medicine, Isehara, Japan

Abstract. Seroprevalence of *Entamoeba histolytica* infection in HIV-infected individuals from Shanghai city, Anhui province, and Henan province, China, was examined by enzyme-linked immunosorbent assay using crude antigen and a recombinant surface antigen, C-Igl, of the parasite. In 215 HIV-infected individuals, the positive rates for these antigens were 12.1% and 7.9%, respectively; these rates were significantly higher than the rates of 3.1% and 0.5%, respectively, in 191 patients with gastrointestinal symptoms who were not infected with HIV. There was no significant difference in seropositivity to *E. histolytica* between men and women. Seropositivity in HIV-infected individuals was higher in patients with a $CD4^+$ T cell count of < 200/μL. This is the first report showing a higher seroprevalence of *E. histolytica* infection in HIV-infected patients in China. Our results also suggest that HIV infection is a risk factor for infection with *E. histolytica*.

Amebiasis caused by infection with *Entamoeba histolytica* is one of the most problematic parasitic diseases in developing and developed countries.[1] *E. histolytica* infection is also related to diarrhea in patients with HIV infection and AIDS, as well as those infected with opportunistic protozoan parasites such as *Cryptosporidium* spp., *Isospora belli*, and microsporidia.[2] Recently, a high prevalence of *E. histolytica* infection has been reported in HIV/AIDS patients in Japan and Taiwan.[3–5] In China, it is estimated that 650,000 individuals are infected with HIV, and HIV/AIDS is becoming a major public health problem (http://data.unaids.org/Media/PressReleases03/PR_china_060125_en.pdf). However, there is no information concerning the correlation between *E. histolytica* and HIV infections, and the prevalence of *E. histolytica* and *E. dispar*, which is morphologically indistinguishable from *E. histolytica* but is non-pathogenic, is not known in China. Because *E. dispar* is non-invasive, detection of high specific antibody titers indicates possible infection with *E. histolytica*.[6] Therefore, this preliminary study was undertaken to estimate the seroprevalence of *E. histolytica* infection in HIV-infected persons in the Chinese population.

This study was approved by the Ethics Committees of Fudan University School of Medicine and Shanghai Public Health Center, which is affiliated with Fudan University. A total of 466 peripheral blood samples were collected from June to August 2005. Samples from 215 individuals with HIV/AIDS were obtained at Shanghai Public Health Center and at treatment centers for AIDS in Henan province and Anhui province. The subjects were selected randomly, but all were receiving anti-retroviral therapy. Symptomatic gastrointestinal complaints or disorders were not recorded at the time the blood samples were collected. For comparison, serum samples from 191 individuals with gastrointestinal symptoms but no HIV infection were obtained at Huashan Hospital, Shanghai. Gastrointestinal symptoms in these individuals were caused by acute gastritis, giant hypertrophic gastropathy, gastric ulcer, duodenal ulcer, acute hemorrhagic necrotizing enteritis, ulcerative colitis, and intestinal obstruction, but many cases did not have a final diagnosis. No specific pathogens have been detected in culture or microscopy of stool samples examined to date. Serology to HIV was examined using a Cambridge Biotech HIV1 Western blot kit (Cambridge Biotech Corp, Rockville, MD). Serum samples from 60 healthy individuals without a history of amebiasis were used as negative controls. All serum samples were inactivated at 56°C for 30 minutes and stored at –30°C or –80°C until use.

Anti-*E. histolytica* serology was examined by enzyme-linked immunosorbent assay (ELISA) using crude *E. histolytica* antigen and the recombinant fragment of C terminus of intermediate subunit of galactose and *N*-acetyl-D-galactosamine-inhibitable lectin of *E. histolytica* (C-Igl), as previously described.[7] The cut-off for a positive result was defined as an ELISA value > 3 SD above the mean for healthy negative controls. Optical density (OD) values were plotted and analyzed using Prism ver. 4.0, and statistical analysis was performed with Stata ver. 7.0. A descriptive exploratory analysis of the data was performed to assess the distribution variables in the HIV-infected and HIV-uninfected groups. Categorical variables were compared using a Pearson χ^2 test.

Quantification of $CD4^+$ lymphocytes in individuals with HIV/AIDS was performed by cytofluorometry using a MultiTEST CD3 FITC/CD8 PE/CD45 PerCP/ CD4 APC (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions.

ELISA reactivities of sera from HIV-infected and HIV-uninfected patients are shown in Figure 1. The cut-off OD values were 0.412 for crude antigen and 0.402 for C-Igl antigen. In HIV-infected individuals, seropositivity to these antigens was 12.1% and 7.9%, respectively (Table 1). These values were significantly higher than those for HIV-uninfected individuals, which were 3.1% and 0.5%, respectively ($P < 0.005$ and $P < 0.001$, respectively). There was no significant difference in seropositivity to *E. histolytica* between HIV-infected men and women. Positive serology to C-Igl antigen in HIV-infected individuals was similar (6.8%–9.1%) in every age category, except for patients < 20 years old, for whom there were a limited number of samples. Seropositivity to C-Igl in HIV-infected individuals from Shanghai was higher

* Address correspondence to Xunjia Cheng, Department of Medical Microbiology and Parasitology, Fudan University School of Medicine, Shanghai 200032, China. E-mail: xjcheng@shmu.edu.cn

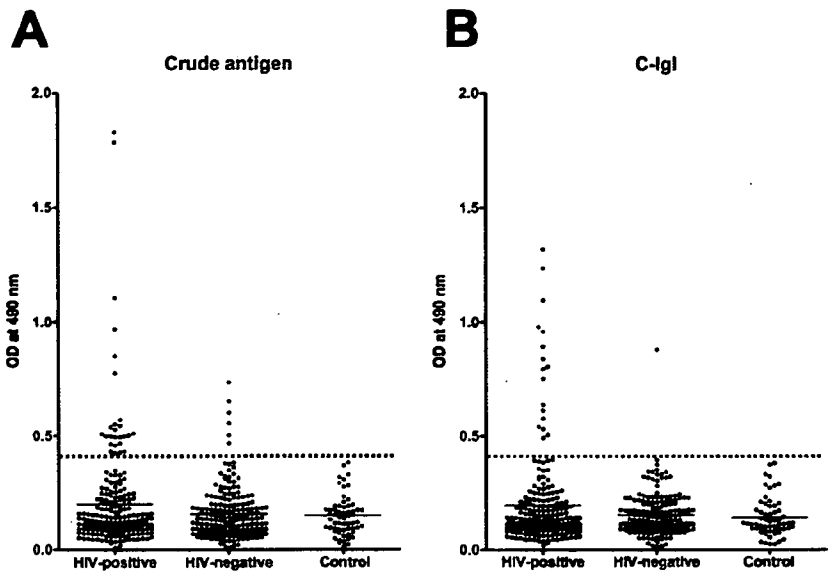

FIGURE 1. ELISA reactivities of sera from HIV/AIDS patients against crude antigen (**A**) and C-Igl (**B**). ELISA plates were coated with 1 μg per well of crude antigen or 100 ng per well of C-Igl. Serum samples from HIV/AIDS patients (HIV-positive, $N = 215$); patients with gastrointestinal symptoms, but not infected with HIV (HIV-negative, $N = 191$); and healthy controls without a history of amebiasis (control, $N = 60$) were used at 1:400 dilution. Horizontal bars indicate the arithmetic means of the groups and the dashed lines indicate the cut-off values.

than those in samples from Anhui and Henan, but the differences were not significant ($P = 0.140$ and $P = 0.122$, respectively). Seroprevalence to *E. histolytica* was higher in patients with a CD4⁺ T-cell count of < 200/μL compared with those with a count of ≥ 200/μL ($P < 0.005$). Stool samples from patients with positive serology to *E. histolytica* were obtained and examined microscopically using a direct smear and subsequent formalin-ether sedimentation. However, we were unable to detect *E. histolytica*/*E. dispar*, and no obvious symptoms of amebiasis were apparent at the time of serum collection.

This is the first report showing higher seroprevalence of *E. histolytica* infection in HIV/AIDS patients in China. In Japan, it is well known that *E. histolytica* infection is common in homosexual men.[8,9] Ohnishi and others[3] have reported that, in 58 patients (including 55 men) with this infection from three large cities in Japan, 56% of the men were homosexual, and antibodies to HIV were detected in 45% of tested pa-

TABLE 1
Seropositivity to *E. histolytica* in HIV/AIDS patients in China

Characteristics	HIV-positive			HIV-negative with gastrointestinal symptoms			Healthy controls		
	No. of samples	Anti-*E. histolytica* antibody-positive (%)		No. of Samples	Anti-*E.histolytica* antibody-positive (%)		No. of samples	Anti-*E. histolytica* antibody-positive (%)	
		Crude Ag	C-Igl		Crude Ag	C-Igl		Crude Ag	C-Igl
Total	215	26 (12.1)	17 (7.9)	191	6 (3.1)	1 (0.5)	60	0 (0)	0 (0)
Sex									
Male	144	18 (12.5)	11 (7.6)	102	6 (5.9)	1 (1.0)	35	0 (0)	0 (0)
Female	71	8 (11.3)	6 (8.5)	89	0 (0)	0 (0)	25	0 (0)	0 (0)
Age (years)									
< 20	3	0 (0)	0 (0)	0	0 (0)	0 (0)	0	0 (0)	0 (0)
20–29	11	3 (27.3)	1 (9.1)	13	0 (0)	0 (0)	60	0 (0)	0 (0)
30–39	66	8 (12.1)	5 (7.6)	87	1 (1.1)	1 (1.1)	0	0 (0)	0 (0)
40–49	80	11 (13.8)	7 (8.8)	61	3 (4.9)	0 (0)	0	0 (0)	0 (0)
50–59	44	3 (6.8)	3 (6.8)	22	2 (9.1)	0 (0)	0	0 (0)	0 (0)
≤ 60	11	1 (9.1)	1 (9.1)	8	0 (0)	0 (0)	0	0 (0)	0 (0)
District									
Shanghai	81	12 (14.8)	10 (12.3)	73	2 (2.7)	0 (0)	20	0 (0)	0 (0)
Anhui	86	12 (14.0)	5 (5.8)	2	1 (50.0)	0 (0)	12	0 (0)	0 (0)
Henan	48	2 (4.2)	2 (4.2)	3	0 (0)	0 (0)	14	0 (0)	0 (0)
Others	0	0 (0)	0 (0)	113	3 (2.7)	1 (0.9)	14	0 (0)	0 (0)
CD4⁺ lymphocytes/μL									
< 200	64	11 (17.2)	11 (17.2)	NA	NA	NA	NA	NA	NA
≤ 200	151	15 (9.9)	6 (4.0)	NA	NA	NA	NA	NA	NA

NA, not applicable.

tients. Recently, a similar trend has been found in HIV-infected persons in Taiwan.[10] In contrast, in Mexico, where both *E. histolytica* and *E. dispar* are endemic, *E. histolytica* prevalence is similar in HIV/AIDS patients and uninfected patients, although the prevalence of *E. dispar* is higher in HIV/AIDS patients.[11] In this study, the absence of a difference in seropositivity to *E. histolytica* between male and female HIV patients suggests that transmission of *E. histolytica* was not caused by homosexual male activity. Indeed, most transmission of HIV in China is thought to be through drug injection, commercial sex, and transfusion (http://data.unaids.org/Media/Press-Releases03/PR_china_060125_en.pdf). Only one HIV-infected man with positive serology for *E. histolytica* was confirmed to be homosexual in this study.

The prevalence of *E. histolytica* and *E. dispar* infections in China is not well understood. A survey in two villages in Shandong province from 1977 to 1984 showed an infection rate of *E. histolytica*/*E. dispar* of 6.4% using microscopy of direct smears of stool samples, with incidences of amebic dysentery and liver abscess of 2.8% and 0.9%, respectively.[12] Another survey in a village in Hebei province in 1985 showed a positive serology rate of 13.2% and a positive rate for *E. histolytica*/*E. dispar* in stool of 9.4%.[13] These reports suggest that *E. histolytica* infection may be endemic in rural areas of China. However, it has been reported that the prevalence of *E. histolytica*/*E. dispar* infection in Henan and Anhui provinces is 0.59% and 0.57%, respectively, using direct microscopy of stool samples.[14] Furthermore, the prevalence of *E. histolytica*/*E. dispar* infection in Shanghai has recently been estimated to be < 0.1% (Xu and others, personal communication). Because microscopy has low sensitivity and specificity for detection of amebiasis, it is possible that these prevalence rates are underestimates. However, the low seroprevalence to *E. histolytica* in non-HIV patients from Shanghai in this study is in good agreement with these data. In addition, our previous observation that two of three *E. histolytica*/*E. dispar* isolates obtained from patients with diarrhea in Shanghai were not *E. histolytica* but *E. dispar* also supports the low seropositivity in the non-HIV group.[15] Therefore, it is likely that the higher seroprevalence among HIV-infected persons is related to HIV infection and characteristics, rather than background seroprevalence because of inclusion of subjects from different districts. However, the reason why seropositivity in HIV patients from Shanghai was higher than in HIV patients from the other two provinces is unclear.

In China, HIV/AIDS patients with diarrhea are commonly treated with metronidazole, trimethoprim, and sulfamethoxazole, even if pathogenic protozoan parasites are not detected in the stool. Therefore, it is likely that the positive serology may be caused by past infection with *E. histolytica*.

It is unclear whether HIV infection is a risk factor for *E. histolytica* infections.[10,11,16] However, the higher seropositivity in patients with a $CD4^+$ cell count of < 200/μL in this study suggests that a deficiency in cellular immunity caused by HIV affects the chance of infection with *E. histolytica*. This observation is in accordance with a study in the United States, which estimated the risk ratios of *E. histolytica*/*E. dispar* infection in HIV-infected patients with $CD4^+$ T cell counts of 0–99 and 100–199/μL as 1.7 and 1.5, respectively, compared with HIV patients with a $CD4^+$ T cell count ≥ 200/μL.[16] It remains unclear how immunodeficiency affects the chance of *E. histolytica* infection. However, it is likely that patients with lower $CD4^+$ T cell counts may be subjected to more testing or that a lower $CD4^+$ T cell count may reflect an increased accumulated risk for amebiasis caused by reactivation of latent infection or increased susceptibility to new infection.[16]

We have recently identified Igl on the surface of trophozoites of *E. histolytica*,[17,18] and it has been shown that an ELISA using C-Igl is more specific than an ELISA using crude antigen.[7] In this study, the seropositive rate in the ELISA using crude antigen was higher than that in the ELISA using C-Igl. All 17 samples that were positive for C-Igl were also positive with crude antigen. The higher seropositivity to crude antigen than to C-Igl antigen seems to be caused by its lower specificity, as previously reported.[7] Therefore, the nine serum samples that were positive with crude antigen but negative with C-Igl may actually be false positives. Overall, our results show that the seroprevalence of *E. histolytica* infection in HIV/AIDS patients in China was 7.9%, which is a higher rate than in HIV-uninfected individuals. A further study will provide a better understanding of the correlation between these infectious diseases.

Received March 13, 2007. Accepted for publication July 2, 2007.

Acknowledgments: The authors thank Xiaozhang Pan for his encouragement of this study.

Financial support: This work was supported by a grant from SMHB (2001411), a Grant-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science, and a grant from the Ministry of Health, Labor and Welfare of Japan.

Authors' addresses: Yi Chen, Bin Yang, and Xunjia Cheng, Department of Medical Microbiology and Parasitology, Fudan University School of Medicine, Shanghai 200032, China, Telephone: 86-21-5423-7359, Fax: 86-21-5423-7122, E-mail: xjcheng@shmu.edu.cn. Yunzhi Zhang, Tangkai Qi, and Hongzhou Lu, Department of Infectious Diseases, Huashan Hospital Affiliated to Fudan University, Shanghai 200041, China, Telephone: 86-21-6248-9999. Hiroshi Tachibana, Department of Infectious Diseases, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan, Telephone: 81-463-93-1121, Fax: 81-463-95-5450, E-mail: htachiba@iss.icc.u-tokai.ac.jp.

REFERENCES

1. Walsh JA, 1986. Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. *Rev Infect Dis 8:* 228–238.
2. DuPont HL, Marshall GD, 1995. HIV-associated diarrhoea and wasting. *Lancet 346:* 352–356.
3. Ohnishi K, Kato Y, Imamura A, Fukayama M, Tsunoda T, Sakaue Y, Sakamoto M, Sagara H, 2004. Present characteristics of symptomatic *Entamoeba histolytica* infection in the big cities of Japan. *Epidemiol Infect 132:* 57–60.
4. Hung CC, Deng HY, Hsiao WH, Hsieh SM, Hsiao CF, Chen MY, Chang SC, Su KE, 2005. Invasive amebiasis as an emerging parasitic disease in patients with human immunodeficiency virus type 1 infection in Taiwan. *Arch Intern Med 165:* 409–415.
5. Hung CC, Chen PJ, Hsieh SM, Wong JM, Fang CT, Chang SC, Chen MY, 1999. Invasive amoebiasis: an emerging parasitic disease in patients infected with HIV in an area endemic for amoebic infection. *AIDS 13:* 2421–2428.
6. World Health Organization, 1997. *Entamoeba* taxonomy. *Bull World Health Organ 75:* 291–294.
7. Tachibana H, Cheng XJ, Masuda G, Horiki N, Takeuchi T, 2004. Evaluation of recombinant fragments of *Entamoeba histolytica* Gal/GalNAc lectin intermediate subunit for serodiagnosis of amebiasis. *J Clin Microbiol 42:* 1069–1074.
8. Takeuchi T, Miyahira Y, Kobayashi S, Nozaki T, Motta SR, Matsuda J, 1990. High seropositivity for *Entamoeba histolytica* infection in Japanese homosexual men: further evidence for the

occurrence of pathogenic strains. *Trans R Soc Trop Med Hyg 84:* 250–251.
9. Takeuchi T, Okuzawa E, Nozaki T, Kobayashi S, Mizokami M, Minoshima N, Yamamoto M, Isomura S, 1989. High seropositivity of Japanese homosexual men for amebic infection. *J Infect Dis 159:* 808.
10. Tsai JJ, Sun HY, Ke LY, Tsai KS, Chang SY, Hsieh SM, Hsiao CF, Yen JH, Hung CC, Chang SC, 2006. Higher seroprevalence of *Entamoeba histolytica* infection is associated with human immunodeficiency virus type 1 infection in Taiwan. *Am J Trop Med Hyg 74:* 1016–1019.
11. Moran P, Ramos F, Ramiro M, Curiel O, Gonzalez E, Valadez A, Gomez A, Garcia G, Melendro EI, Ximenez C, 2005. Infection by human immunodeficiency virus-1 is not a risk factor for amebiasis. *Am J Trop Med Hyg 73:* 296–300.
12. Zheng YL, 1986. An epidemiologic survey on Endoameba histolytica infection. *Zhonghua Liu Xing Bing Xue Za Zhi 7:* 274–276.
13. Zhang TH, 1988. Seroepidemiological study on amoebiasis. *Zhonghua Liu Xing Bing Xue Za Zhi 9:* 217–219.
14. Xu LQ, Yu SH, Xu SH, 1999. *Distribution and Pathogenic Impact of Human Parasites in China.* Beijing: People's Medical Publishing House.
15. Cheng XJ, Tachibana H, Kobayashi S, Kaneda Y, Huang MY, 1993. Pathogenicity of *Entamoeba histolytica* isolates from Shanghai, China. *Parasitol Res 79:* 608–610.
16. Lowther SA, Dworkin MS, Hanson DL, 2000. *Entamoeba histolytica/Entamoeba dispar* infections in human immunodeficiency virus-infected patients in the United States. *Clin Infect Dis 30:* 955–959.
17. Cheng XJ, Tsukamoto H, Kaneda Y, Tachibana H, 1998. Identification of the 150-kDa surface antigen of *Entamoeba histolytica* as a galactose- and N-acetyl-D-galactosamine-inhibitable lectin. *Parasitol Res 84:* 632–639.
18. Cheng XJ, Hughes MA, Huston CD, Loftus B, Gilchrist CA, Lockhart LA, Ghosh S, Miller-Sims V, Mann BJ, Petri WA Jr, Tachibana H, 2001. Intermediate subunit of the Gal/GalNAc lectin of *Entamoeba histolytica* is a member of a gene family containing multiple CXXC sequence motifs. *Infect Immun 69:* 5892–5898.

Parasitol Res (2007) 102:103–110
DOI 10.1007/s00436-007-0736-z

ORIGINAL PAPER

Differences in protein profiles of the isolates of *Entamoeba histolytica* and *E. dispar* by surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) ProteinChip assays

Asao Makioka · Masahiro Kumagai · Seiki Kobayashi · Tsutomu Takeuchi

Received: 4 August 2007 / Accepted: 17 August 2007 / Published online: 11 September 2007

Abstract Surface-enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF MS) ProteinChip assays with weak cationic exchange chips were used for protein profiling of different isolates of *Entamoeba histolytica* and *E. dispar*. When SELDI-TOF MS spectra of cell lysates from *E. histolytica* strain HM-1:IMSS were compared with those from four other laboratory strains (200:NIH, HK-9, DKB, and SAW755CR) grown under the same culture conditions, different peak patterns of SELDI-TOF MS were observed among these strains, independent of their zymodeme types. Similarly, five Japanese isolates of *E. histolytica* grown under the same culture conditions revealed different peak patterns among themselves. The SELDI-TOF MS spectra of cell lysates from two isolates of *E. dispar* strain AS16IR and CYNO 09:TPC showed the presence of peaks specific for *E. dispar* isolates and the absence of peaks common to *E. histolytica* isolates. This is not only the first use of SELDI-TOF MS ProteinChip technology for protein profiling of different strains of *Entamoeba* but also the use for parasitic protozoa. The SELDI-TOF MS spectra show a realistic view of proteins with a biological status of *E. histolytica* and *E. dispar* isolates, contributing to show their phenotypic differences of proteins and provide a unique means of distinguishing them.

A. Makioka (✉) · M. Kumagai
Department of Tropical Medicine,
Jikei University School of Medicine,
3-25-8 Nishi-shinbashi, Minato-ku,
Tokyo 105-8461, Japan
e-mail: makioka@jikei.ac.jp

S. Kobayashi · T. Takeuchi
Department of Tropical Medicine and Parasitology,
Keio University School of Medicine,
35 Shinanomachi, Shinjuku-ku,
Tokyo 160-8582, Japan

Introduction

Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) ProteinChip assay is a relatively recent technology for exploring proteomes combining chromatography and mass spectrometry (Hutchens and Yip 1993; Issaq et al. 2002). The ProteinChip arrays contain sample spots of 1 or 2 mm diameter, with each chip having a different surface chemistry. These may be chemical (e.g., ionic, hydrophobic, hydrophilic) or biochemical (antibody, receptor, deoxyribonucleic acid [DNA], etc.) and are designed to capture proteins of interest and then analyzed directly on the SELDI-TOF mass spectrometer. The ProteinChip technology has been used for protein profiling and biomarker discovery for diseases such as cancer, neurological disorders, and pathogenic organisms including human African trypanosomiasis (Issaq et al. 2002; Papadopoulos et al. 2004).

Entamoeba histolytica, a protozoan parasite, is responsible for an estimated 40–50 million cases of amebic colitis and liver abscess (WHO/PAHO/UNESCO 1997). There are a large number of isolates of *E. histolytica* that differ in their phenotypes. Although genetic diversity of isolates of *E. histolytica* has been extensively studied, there are few reports on differences in their protein profiles as the phenotype. In this study, we used the SELDI-TOF MS ProteinChip

assays to examine protein profiles of different isolates of *E. histolytica*. We also compared protein profiles of *E. histolytica* with those of nonpathogenic *E. dispar*.

Materials and methods

Five laboratory strains and five Japanese isolates of *E. histolytica* and two isolates of *E. dispar* were used in this study (Table 1). The Japanese isolates of *E. histolytica* were obtained from clinical specimens collected from amebiasis patients in Japan. Axenic in vitro cultures were established and maintained in Diamond's BI-S-33 medium as previously described (Diamond et al. 1978). The two *E. dispar* isolates included one human isolate and one nonhuman primate isolate. The *E. dispar* trophozoites were cultivated axenically in newly designed medium (Kobayashi et al. 2005) with a modification of replacement of gluconic acid with maltose (Kobayashi et al. manuscript in preparation). All cases with intestinal amebiasis or liver abscess were diagnosed by microscopic demonstration of trophozoites or cysts in stool or of trophozoites in liver aspirates, respectively. Zymodeme analysis (Sargeaunt 1988) and polymerase chain reaction analysis (Tachibana et al. 1991; Cheng et al. 1993) of the amoeba isolates were performed to characterize them.

Trophozoites of *E. histolytica* and *E. dispar* in log-phase culture were harvested by centrifugation at 400×*g* for 5 min and washed by centrifugation three times in phosphate-buffered saline and stored in −80°C until use. The pellets of ameba were dissolved in 2% CHAPS (Sigma-Aldrich, St Louis, MO), 5 mM Tris–HCl buffer, pH 8.0 containing proteinase cocktail (Sigma-Aldrich) with 140 μM E-64 by vortexing. After centrifugation at 15,000×*g* for 15 min, the resulting supernatants were used as whole-cell lysates. Protein concentration was determined by Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

One microgram of cell extract diluted into 4 μl of starting buffer was spotted onto each ProteinChip array (originally Ciphergen Biosystems, Fremont, CA; now Bio-Rad Laboratories) with CM10 (weak cation exchanger: Carboxylate). The chips were then incubated at room temperature for 20 min on a shaker. Nonbound proteins and other contaminants were washed from the CM10 ProteinChip arrays with a buffer of 0.1 M ammonium acetate, pH 4.0, three times. Finally, all chips were washed with MilliQ water to remove interfering salts and detergents. After drying, 0.5 μl of saturated energy-absorbing molecule solution (sinapinic acid in acetonitrile [v/v] and trifluoroacetic acid [v/v]) was added twice, and the chips were allowed to air dry. Mass spectrometry analysis was performed by time-of-flight mass spectrometry in a PBS-II mass reader (Ciphergen Biosystems). Spectra were collected using an average 80 nitrogen laser shots. Spectrum analysis was performed using the ProteinChip software version 2.1b (Ciphergen Biosystems). The optimal detection size range was set between 2,000 and 20,000 Da because the system is most effective at profiling low-molecular-weight proteins (i.e., <20 kDa; Issaq et al. 2002). The intensity of each

Table 1 *Entamoeba histolytica* and *E. dispar* isolates used in this study

Isolate	Isolation		Clinical diagnosis	Serology[a] (S)/PCR[b] (P)	Zymodeme[c]
	Location	Year			
E. histolytica					
HM-1:IMSS	Mexico	1967	Dysentery	NA[d]	II
200:NIH	USA	1949	Dysentery	NA	II
HK-9	Korea	1951	Dysentery	NA	II
DKB	UK	1924	Dysentery	NA	II
SAW755CR	UK	1979	Hematophagous trophozoites in feces	NA	XIV
KU43	Japan	2002	Colitis	S +	II
KU46	Japan	2004	Colitis	S +	XXI
KU2	Japan	1988	Colitis	S +	XIX
KU38	Japan	2002	Asymptomatic	S +	II
KU14	Japan	1999	Asymptomatic	S +	XIX
E. dispar					
AS16IR	Iran	1997	Abdominal pain	P +	I
CYNO 09:TPC	Philippines	1994	NA	P +	I

[a] Serology was done by enzyme-linked immunosorbent assay, gel diffusion test, and/or indirect fluorescent-antibody test.
[b] PCR analysis using two sets of oligonucleotide primers each (p11 plus p12 and p13 plus p14, respectively) for amplification of the DNAs of *E. histolytica* and *E. dispar* was performed.
[c] Zymodems type I and III are classified as *E. dispar*.
[d] *NA* Not available

of the peaks to be quantified was determined according to externally calibrated standards (Ciphergen Biosystems). According to the manufacturer, the mass accuracy of the spectrometer is 0.1%. The raw intensity data were normalized using the total ion current of *m/z* between 2,000 and 20,000 for all study sample profiles.

The mean±SE of the intensity of each peak in SELDI-TOF MS spectra from three independent cultures was calculated for all the isolates. The percentages of the number of peaks whose intensities were comparable to between the two isolates were shown as percentage similarity of the peak pattern. From these values, a dendrogram was generated by the unweighted pair-group method with arithmetic mean (UPGMA).

Results

Reproducibility of SELDI-TOF MS spectra tested with *E. histolytica* HM-1:IMSS

SELDI-TOF MS spectra of the whole cell lysates of *E. histolytica* HM-1:IMSS as a standard strain from three independent cultures are shown in Fig. 1. The peak patterns were almost reproducible. The two groups of peaks in which 4.305 and 8.274 kDa as major peaks were detected in a mass range of 3–15 kDa.

Comparison of SELDI-TOF MS spectra between *E. histolytica* HM-1:IMSS and other laboratory strains

SELDI-TOF MS spectra of *E. histolytica* strain HM-1:IMSS were compared with those from four other laboratory strains, 200:NIH, HK-9, DKB, and SAW755CR, grown under the same culture conditions. As shown in Fig. 2, peaks of HM-1:IMSS in the mass range of 3–6 kDa were significantly higher than those of the other four strains. The peaks of 6–8 kDa were low in all the strains. In the mass range of 8–10 kDa, a peak of 8.274 kDa of HM-1:IMSS was significantly higher than those of the other four strains, whereas peaks of 8.568, 8.791, and 9.267 kDa of HM-1:IMSS were lower than those of the other four strains. In addition, the peak of 9.267 kDa revealed different intensities among the other four strains. There were low peaks in the range of 10–15 kDa in all the strains.

Comparison of SELDI-TOF MS spectra between *E. histolytica* HM-1:IMSS and Japanese isolates

SELDI-TOF MS spectra of *E. histolytica* strain HM-1:IMSS were compared with those from five Japanese isolates, KU43, KU46, KU2, KU38, and KU14. As shown in Fig. 3, only KU2 showed lower peaks than HM-1:IMSS in the range of 3–6 kDa. The peak of 5.423 kDa, which was little seen in the laboratory strains except HM-1:IMSS, was detected in the Japanese isolates except KU2. The intensity of the major peak of 4.305 kDa was comparable to or higher than that of HM-1:IMSS in the Japanese isolates except KU2. The Japanese isolates also showed low peaks in the range of 6–8 kDa, although several peaks of KU2 were higher than those of the other four Japanese isolates. In the range of 8–10 kDa, the peak patterns of KU43, KU38, and KU14 were relatively similar to that of HM-1:IMSS, whereas a high peak of 9.267 kDa was observed only in KU2. The peak of 8.568 kDa in KU46 and KU2 was significantly higher than those of HM-1:IMSS and other Japanese isolates. No higher peaks were detected in the range of 10–15 kDa of all the Japanese isolates like the laboratory strains of *E. histolytica*.

Comparison of SELDI-TOF MS spectra between *E. histolytica* HM-1:IMSS and *E. dispar* isolates

SELDI-TOF MS spectra of *E. histolytica* HM-1:IMSS grown in the medium for *E. dispar* were similar to those grown in BI-S-33 medium (Fig. 1; data not shown). When SELDI-TOF MS spectra of HM-1:IMSS were compared with those from *E. dispar* isolates, AS16IR and CYNO 09:TPC, the

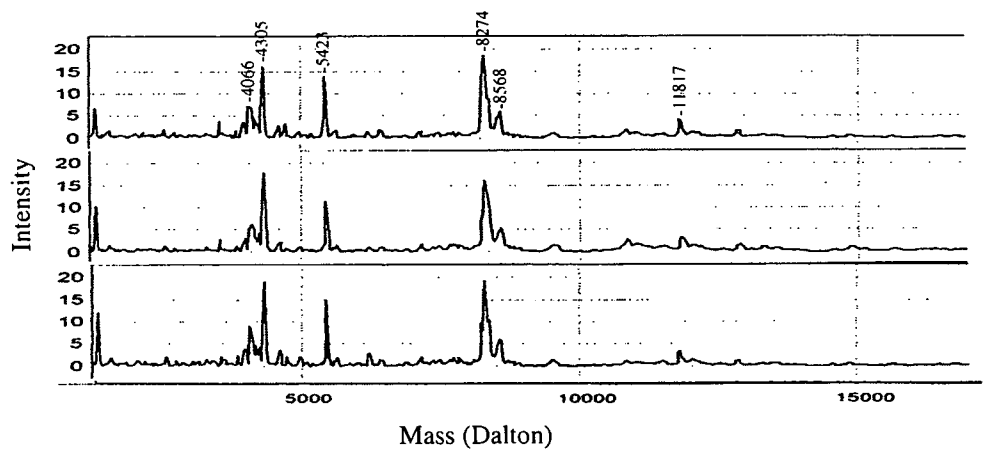

Fig. 1 SELDI-TOF MS spectra of *E. histolytica* strain HM-1:IMSS from three independent cultures. The molecular masses are shown above the peaks

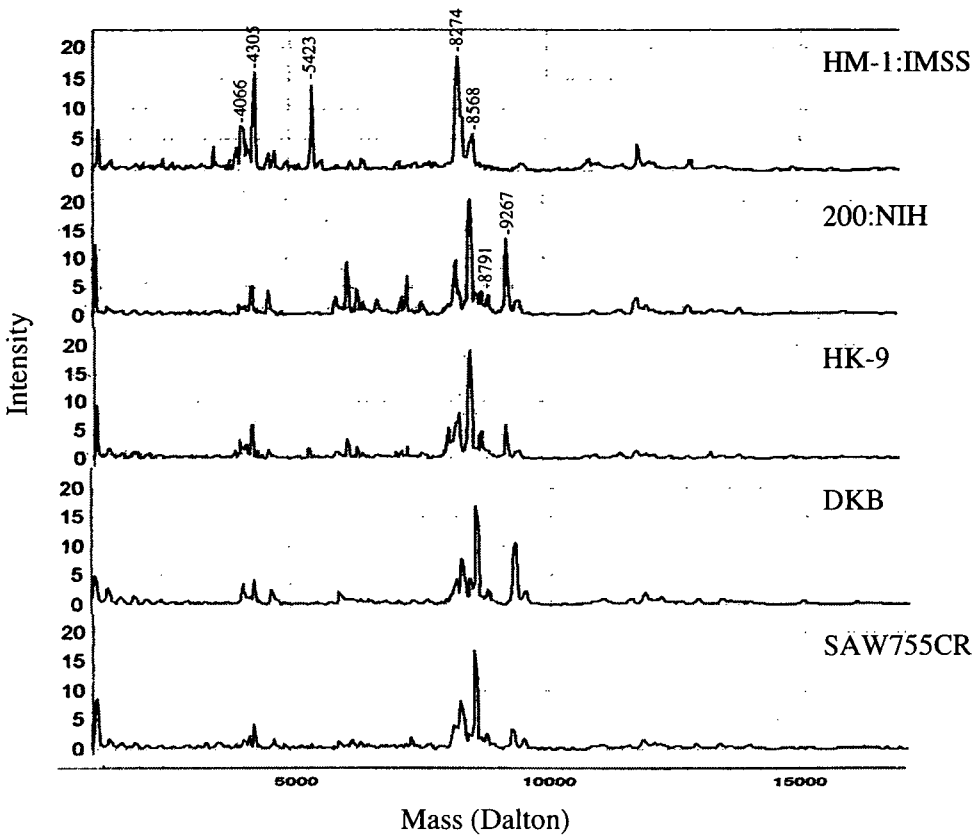

Fig. 2 Representative SELDI-TOF MS spectra of five laboratory strains of *E. histolytica*. The molecular masses are shown above the peaks

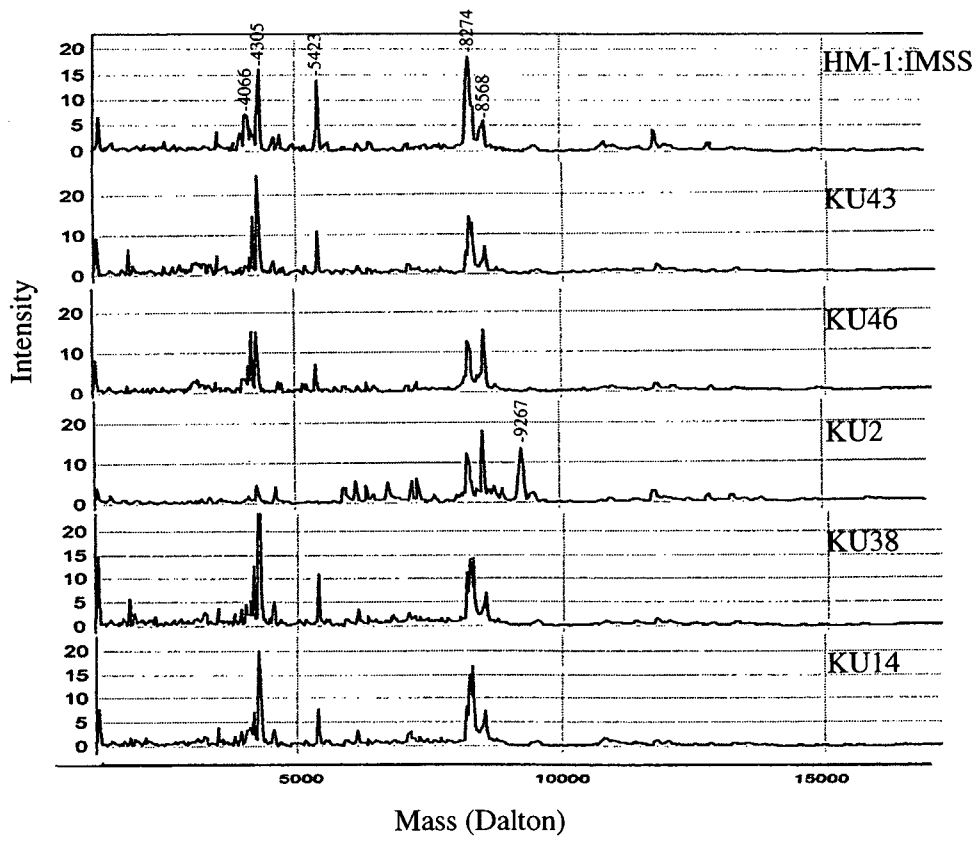

Fig. 3 Representative SELDI-TOF MS spectra of five Japanese isolates of *E. histolytica*. The molecular masses are shown above the major peaks

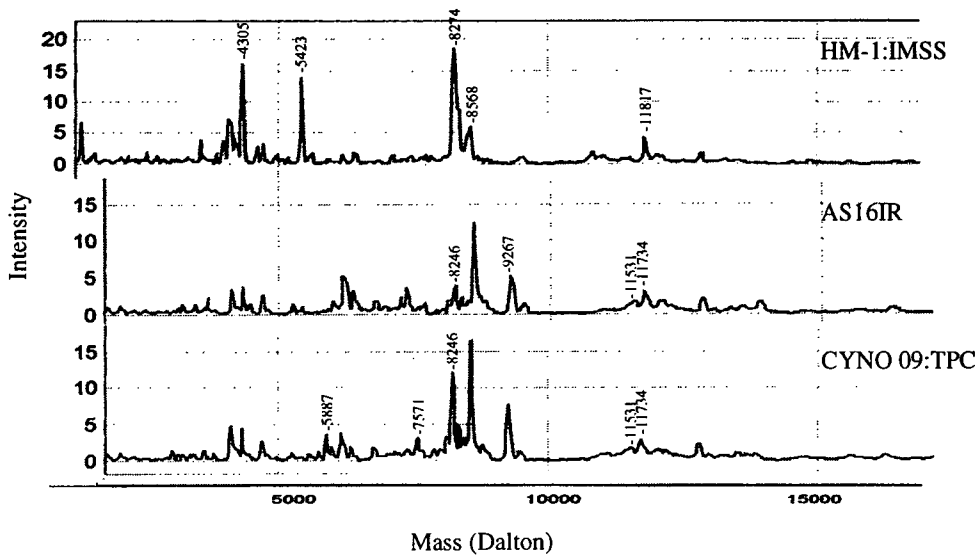

Fig. 4 Representative SELDI-TOF MS spectra of *E. dispar* isolates. The molecular masses are shown above the major peaks

peaks of 4.305 and 5.423 kDa were significantly lower than those of HM-1:IMSS, which was similar to the *E. histolytica* laboratory strains except HM-1:IMSS. The peak of 5.887 kDa was detected only in CYNO 09:TPC. The peaks of AS16IR and CYNO 09:TPC were mostly low in the range of 6–8 kDa, and the peak of 7.571 kDa was observed only in CYNO 09:TPC. In the range of 8–10 kDa, the major peak of 8.274 kDa common to *E. histolytica* isolates was not seen in either isolate of *E. dispar*, but the peak of 8.246 kDa was observed. Furthermore, like the *E. histolytica* laboratory strains except HM-1:IMSS, the peak of 8.568 kDa was significantly higher than that of HM-1:IMSS. The peak of 9.267 kDa, which was not seen in HM-1:IMSS, was observed in both isolates. In the range of 10–15 kDa, two peaks of 11.531 and 11.734 kDa not detected in *E. histolytica* were detected in both isolates of *E. dispar* (Fig. 4).

Similarity of peak patterns by SELDI-TOF MS among *E. histolytica* and *E. dispar* isolates and a dendrogram by the UPGMA method

The HM-1:IMSS showed 38.4 (200:NIH) to 69.2% (KU38 and KU14) similarity against other *E. histolytica* isolates, and KU38 and KU14 showed the highest similarity of 96.9% (Fig. 5). *E. dispar* AS16IR and CYNO 09:TPC showed 81.5% similarity to each other and were most associated with *E. histolytica* DKB, showing 72.3% similarity. The dendrogram representing relations among *E. histolytica* and

Fig. 5 Similarity of protein profiles among *E. histolytica* and *E. dispar* isolates. Percentage similarities were determined as described in Materials and methods

	HM-1:IMSS	200:NIH	HK-9	DKB	SAW755CR	KU43	KU46	KU2	KU38	KU14	AS16IR	CYNO09:TPC
HM-1:IMSS												
200:NIH	38.4											
HK-9	50.8	81.5										
DKB	53.8	76.9	81.5									
SAW755CR	44.6	70.8	83.1	87.7								
KU43	56.9	50.8	64.6	63.1	58.5							
KU46	49.2	56.9	70.8	70.8	63.1	67.7						
KU2	41.5	83.1	75.4	78.5	70.8	44.6	61.5					
KU38	69.2	55.4	72.3	70.8	67.7	84.6	83.1	60.0				
KU14	69.2	58.5	73.8	80.0	72.3	83.1	83.1	63.1	96.9			
AS16IR	38.5	61.5	61.5	72.3	61.5	47.7	44.6	55.4	58.5	56.9		
CYNO09:TPC	29.2	53.8	58.5	72.3	63.1	44.6	46.2	43.1	47.7	55.4	81.5	

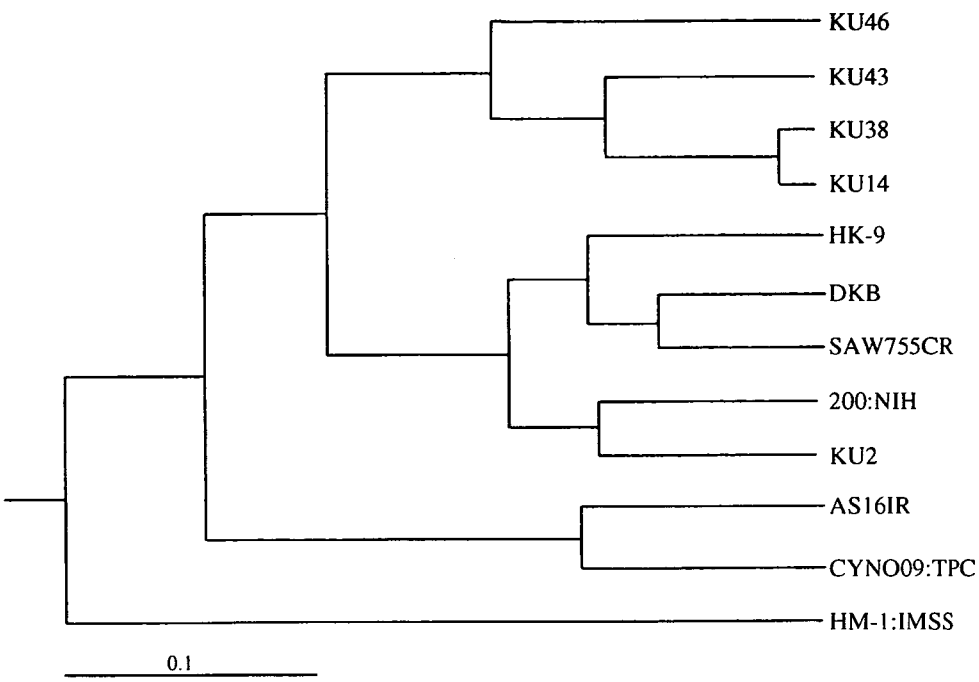

Fig. 6 Dendrogram representing relations among *E. histolytica* and *E. dispar* isolates by the UPGMA method

E. dispar isolates indicates that HM-1:IMSS is in an independent clade separated from other *E. histolytica* isolates, and two isolates of *E. dispar* were in one clade (Fig. 6). In two other clades, one includes KU46, KU43, KU38, and KU14, and the other includes HK-9, DKB, SAW755CR, 200:NIH, and KU2. DKB and SAW755CR, and 200:NIH and KU2, as well as KU38 and KU14 were included in the same clades, respectively (Fig. 6).

Discussion

ProteinChip technology is a new technology used to study proteomic profiles in biological samples, such as serum, cerebrospinal fluid, and cell or tissue extracts (Merchant and Weinberger 2000; Wulfkuhle et al. 2001; Carrette et al. 2003; Luo et al. 2003; Carlson et al. 2004) and is used in biomarker discovery and protein profiling (Ball et al. 2002; Choe et al. 2002; Yasui et al. 2003; Hayman and Przyborski 2004). With regard to parasitic protozoa, this technology was first used to detect antigens in sera of patients with African trypanosomiasis as a novel diagnostic method (Papadopoulos et al. 2004).

The heterogeneity of several DNA loci, including protein-coding sequences, has been extensively characterized among *E. histolytica* and *E. dispar* isolates (Clark and Diamond 1993; Ghosh et al. 2000; Zaki and Clark 2001; Zaki et al. 2002; Haghighi et al. 2002; Haghighi et al. 2003), and the overall genomic diversity among them has also been reported (Shah et al. 2005). However, there have been few reports on protein profiling among isolates of the two *Entamoeba* species. In this study, we used SELDI-TOF MS ProteinChip technology to identify protein patterns in different isolates of *E. histolytica* and showed phenotypic heterogeneity of proteins among them even under identical culture conditions. The results clearly indicate differences in SELDI-TOF MS spectra between the HM-1:IMSS and the other four laboratory strains and also between the HM-1:IMSS and the Japanese isolates, independent of their zymodemes. The HM-1:IMSS, which is most widely used as a standard strain, was in a separate clade in the dendrogram from the other *E. histolytica* isolates. Although the reason for this remains unclear, it should be taken into consideration when *E. histolytica* HM-1:IMSS as a standard strain is compared with other *Entamoeba* species. The percentage similarity in peak patterns among the isolates did not distinguish *E. dispar* from *E. histolytica*. It is clear from comparison of protein profiles between *E. histolytica* and *E. dispar* that there are peaks specific for each species of amoeba. These could be useful markers for distinguishing the two species, although the number of *E. dispar* isolates used in this study is limited.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been the most commonly used method for proteomic analysis (GÖrg et al. 2004), but the need for protein staining and the subsequent sample handling limits the sensitivity of the overall approach (Issaq et al. 2002). The 2D-PAGE is also laborious, difficult to automate, has poor resolution for extreme masses and extreme acidic or basic proteins, and requires large amount of starting material. The 2D-PAGE has recently been used for proteome of *E. histolytica* HM-1:IMSS trophozoites (Leitsch et al. 2005).

If 2D-PAGE is used for analysis of a number of different isolates of *E. histolytica* and *E. dispar*, it would be very laborious, time consuming, and difficult to analyze. In this respect, SELDI-TOF MS ProteinChip technique has an advantage over 2D-PAGE.

There is a wide variety of clinical manifestations observed among individuals infected with *E. histolytica* and/or *E. dispar*. What determines these differences remains unclear. In this respect, studies on phenotypic differences in proteins other than genetic heterogeneity are important and would contribute to resolving the question because the proteome, compared with the genome, provides a more realistic view of a biological status and is, therefore, expected to be more useful than gene analysis for evaluating disease presence and progression and response to treatment (Engwegen et al. 2006). Thus, proteomics can bridge the gap between the genome sequence and cellular behavior.

Finally, SELDI-TOF MS ProteinChip technique was successfully used to analyze different isolates of *E. histolytica* and *E. dispar*. Using the different array surfaces, a complete picture of each strain of *Entameoba* may be drawn, and thus a better set of protein fingerprint profiles for each strain would be provided. Like the successful application of this technology to bacterial proteomes (Barzaghi et al. 2004), the results show the usefulness of ProteinChip technology for studying the proteomics of parasitic protozoa as well, alone or in combination with other technologies.

Acknowledgments We thank N Watanabe for his valuable discussion with us, T Obata for SELDI-TOF MS ProteinChip analysis, and T Yamashita for her technical assistance. This work was supported by a Health Science Research Grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan.

References

Ball G, Mian S, Holding F, Allibone RO, Lowe J, Ali S, Li G, McCardle S, Ellis IO, Creaser C, Rees RC (2002) An integrated approach utilizing artificial neural networks and SELDI mass spectrometry for the classification of human tumours and rapid identification of potential biomakers. Bioinformatics 18:395–404

Barzaghi D, Isbister JD, Lauer KP, Born TL (2004) Use of surface-enhanced laser desorption/ionization-time of flight to explore bacterial proteomes. Proteomics 4:2624–2628

Carlson KA, Ciborowski P, Schellpeper CN, Biskup TM, Shen RF, Luo X, Destache CJ, Gendelman HE (2004) Proteomic fingerprinting of HIV-1-infected human monocyte-derived macrophages: a preliminary report. J Neuroimmunol 147:35–42

Carrette O, Demalte I, Scherl A, Yalkinoglu O, Corthals G, Burkhard P, Hochstrasser DF, Sanchez JC (2003) A panel of cerebrospinal fluid potential biomarkers for the diagnosis of Alzheimer's disease. Proteomics 3:1486–1494

Cheng X-J, Tachibana H, Kobayashi S, Kaneda Y, Huang M-Y (1993) Pathogenicity of *Entamoeba histolytica* isolates from Shanghai, China. Parasitol Res 79:608–610

Choe LH, Dutt MJ, Relkin N, Lee KH (2002) Studies of potential cerebrospinal fluid molecular markers for Alzheimer's disease. Electrophoresis 23:2247–2251

Clark CG, Diamond LS (1993) *Entamoeba histolytica*: a method for isolate identification. Exp Parasitol 77:450–455

Diamond LS, Harlow DR, Cunnick CC (1978) A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans R Soc Trop Med Hyg 72:431–432

Engwegen JYMN, Gast MC, Schellens JHM, Beijnen JH (2006) Clinical proteomics: searching for better tumour markers with SELDI-TOF mass spectrometry. Trends Pharmacol Sci 27:251–259

Ghosh S, Frisardi M, Ramirez-Avila L, Descoteaux S, Sturm-Ramirez K, Newton-Sanchez OA, Santos-Preciado JI, Ganguly C, Lohia A, Reed S, Samuelson J (2000) Molecular epidemiology of *Entamoeba* spp.: evidence of a bottleneck (demographic sweep) and transcontinental spread of diploid parasites. J Clin Microbiol 38:3815–3821

Görg A, Weiss W, Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics 4:3665–3685

Haghighi A, Kobayashi S, Takeuchi T, Masuda G, Nozaki T (2002) Remarkable genetic polymorphism among *Entamoeba histolytica* isolates from a limited geographic area. J Clin Microbiol 40:4081–4090

Haghighi A, Kobayashi S, Takeuchi T, Thammapalerd N, Nozaki T (2003) Geographic diversity among genotypes of *Entamoeba histolytica* field isolates from a limited geographic area. J Clin Microbiol 41:3748–3756

Hayman MW, Przyborski SA (2004) Proteomic identification of biomarkers expressed by human pluripotent stem cells. Biochem Biophys Res Commun 316:918–923

Hutchens TW, Yip TT (1993) New desorption strategies for the mass spectrometric analysis of macromolecules. Rapid Commun Mass Spectrom 7:576–580

Issaq HJ, Veenstra TD, Cornard TP, Felschow D (2002) The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. Biochem Biophys Res Commun 292:587–592

Kobayashi S, Imai E, Haghighi A, Khalifa SA, Tachibana H, Takeuchi T (2005) Axenic cultivation of *Entamoeba dispar* in newly desighed yeast extract-iron–gluconic acid–dihydroxyacetone–serum medium. J Parasitol 91:1–4

Leitsch D, Radauer C, Paschinger K, Wilson IB, Breiteneder H, Scheiner O, Duchene M (2005) *Entamoeba histolytica*: analysis of the trophozoite proteome by two-dimensional polyacrylamide gel electrophoresis. Exp Parasitol 110:191–195

Luo X, Carlson KA, Wojna V, Mayo R, Biskup TM, Stoner J, Anderson J, Gendelman HE, Melendez LM (2003) Macrophage proteomic fingerprinting predicts HIV-1-associated cognitive impairment. Neurology 60:1931–1937

Merchant M, Weinberger SR (2000) Recent advancements in surface enhanced laser desorption/ionization-time of flight-mass spectrometry. Electrophoresis 21:1164–1177

Papadopoulos MC, Abel PM, Stich A, Tarelli E, Bell BA, Planche T, Loosemore A, Saadoun S, Wilkins P, Krishna S (2004) A novel and accurate diagnostic test for human African trypanosomiasis. Lancet 363:1358–1363

Sargeaunt PG (1988) Zymodemes of *Entamoeba histolytica*. In: Radvin JI (ed) Amebiasis: human infection by *Entamoeba histolytica*. Wiley, New York, pp 370–387

Shah PH, MacFarlane RC, Bhattacharya D, Matese JC, Demeter J, Stroup SE, Singh U (2005) Comparative genomic hybridizations of *Entamoeba* strains reveal unique genetic fingerprints that correlate with virulence. Eukaryotic Cell 4:504–515

Tachibana H, Kobayashi S, Takekoshi M, Ihara S (1991) Distinguishing pathogenic isolates of *Entamoeba histolytica* by polymerase chain reaction. J Infect Dis 164:825–826

WHO/PAHO/UNESCO (1997) A consultation with experts on amebiasis. Epidemiol Bull 18:13–14

Wulfkuhle JD, McLean KC, Paweletz CP, Sgroi DC, Trock BJ, Steeg PS, Petricoin EF III (2001) New approaches to proteomic analysis of breast cancer. Proteomics 1:1205–1215

Yasui Y, Pepe M, Thompson ML, Adam BL, Wright GL Jr, Qu Y, Potter JD, Winget M, Thornquist M, Feng Z (2003) A data-analytic strategy for protein biomarker discovery: profiling of high-dimensional proteomic data for cancer detection. Biostatistics 4:449–463

Zaki M, Clark CG (2001) Isolation and characterization of polymorphic DNA from *Entamoeba histolytica*. J Clin Microbiol 39:897–905

Zaki M, Meelu P, Sun W, Clark CG (2002) Simultaneous differentiation and typing of *Entamoeba histolytica* and *Entamoeba dispar*. J Clin Microbiol 40:1271–1276

Seroprevalence of *Entamoeba histolytica* Infection in Female Outpatients at a Sexually Transmitted Disease Sentinel Clinic in Tokyo, Japan

Jun Suzuki[1*], Seiki Kobayashi[2], Ise Iku[1], Rie Murata[1], Yoshitoki Yanagawa[1] and Tsutomu Takeuchi[2]

[1]Division of Clinical Microbiology, Department of Microbiology

Tokyo Metropolitan Institute of Public Health

3-24-1, Hyakunin-cho, Shinjuku-ku

Tokyo 169-0073, Japan

[2]Department of Tropical Medicine and Parasitology

School of Medicine, Keio University

35 Shinanomachi, Shinjuku-ku

Tokyo 160-8582, Japan

*Corresponding author.

Address: Division of Clinical Microbiology, Department of Microbiology

Tokyo Metropolitan Institute of Public Health

3-24-1, Hyakunin-cho, Shinjuku-ku, Tokyo 169-0073, Japan

Tel.: +81-3-3363-3231, Fax: +81-3-3368-4060

E-mail: Jun_Suzuki@member.metro.tokyo.jp

SUMMARY

From 2003 to 2006, we surveyed the seroprevalence of amoebic infection in female outpatients from a gynecologist's office, designated as a sexually transmitted disease sentinel clinic by the Tokyo Metropolitan Government, using an enzyme-linked immunosorbent assay (ELISA). The annual rate of anti-*Entamoeba histolytica* (HM-1:IMSScl6 strain; HM-1) antibody-positive cases as detected by ELISA has increased, and anti-*Chlamydia trachomatis* antibodies were detected in 60%, i.e., 24/40, among these anti-HM-1 antibody-positive individuals, suggesting sexual transmission of *E. histolytica* (EH). We designed an ELISA with better sensitivity using the antigen extracted from the virulence-augmented EH strains (LHM-1 and LLA526 strains) by liver-passaging in hamsters. The average ratios of the S/N value (optical density (OD) of sample/OD of negative control) of ELISA with either the LHM-1 or LLA526 antigen and that of ELISA with the HM-1 antigen increased significantly in intestinal amoebiasis cases with low S/N values compared to the average ratios in amoebic liver abscess cases. In the present study for seroprevalence of *E. histolytica* infection, the sera testing positive with low S/N values (<10) by ELISA with HM-1 antigen exhibited higher S/N values by ELISA using LHM-1 and LLA526 antigens. It is expected to be effective in detecting anti-EH antibodies from such asymptomatic patients who have low antibody titers.

INTRODUCTION

In Japan, until the mid-1970s, amoebiasis was thought to be solely food borne and spread via food contaminated with cysts of *Entamoeba histolytica* (EH). However, in the late 1970s, since amoebiasis was reported among men having sex with men (MSM)

in large cities of the USA, it was recognized as a sexually transmitted disease (STD) (1, 2). In case of Japan, since the beginning of the 1980s, the suspected number of MSM having anti-EH antibodies along with anti-*Treponema pallidum* (TP) antibodies began to increase in densely populated cities (3, 4).

In the data of the National Epidemiological Surveillance of Infectious Diseases, the number of notified cases with amoebiasis has been increasing annually; in 2006, 747 cases were reported, and approximately 90% of the notified cases were male. However, with the spread of amoebiasis, the number of notified female cases has also increased at a slow but steady pace since 1999 (5, 6).

In the present study, by detecting anti-EH (HM-1:IMSScl6 strain; HM-1) antibodies using an enzyme-linked immunosorbent assay (ELISA), we report the seroprevalence of amoebic infection in female outpatients who visited a gynecologist's office in Tokyo, Japan, from 2003 to 2006; this office was designated as an STD sentinel clinic by the Tokyo Metropolitan Government.

Moreover, in this study, we attempted to design an ELISA with better sensitivity; this involved the use of the antigen extracted from the virulence-augmented EH strains by liver-passaging in hamsters. The serum anti-EH antibody titers are low in a majority of asymptomatic amoebiasis cases. Practically, this serological method using LHM-1 and LLA526 antigens was tested on the anti-HM-1 antibody-positive sera in the present surveillance study.

MATERIALS AND METHODS

Study population: This study was conducted at a gynecologist's office in Tokyo, Japan, designated as an STD sentinel clinic by the Tokyo Metropolitan Government. We collected 981 blood samples from female outpatients between 2003 and 2006 (205 in