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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

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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

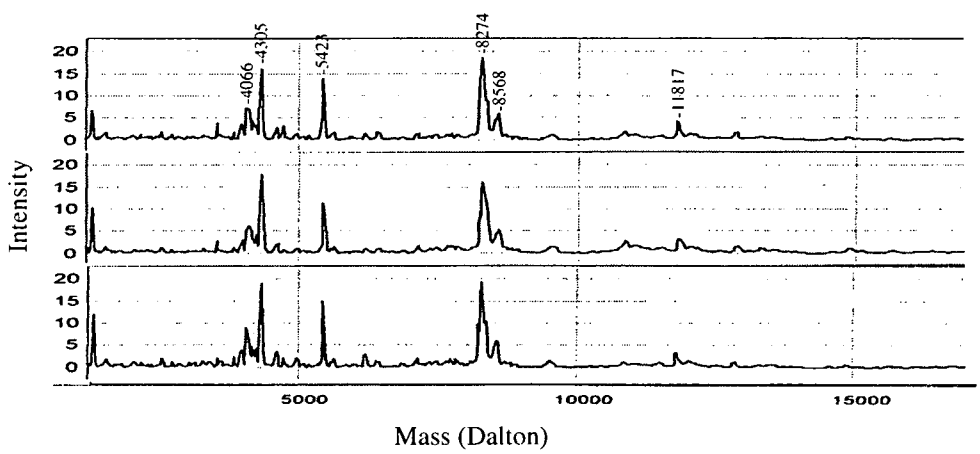

Fig. 1 SELDI-TOF MS spectra of *E. histolytica* strain HM-1:IMSS from three independent cultures. The molecular masses are shown above the peaks

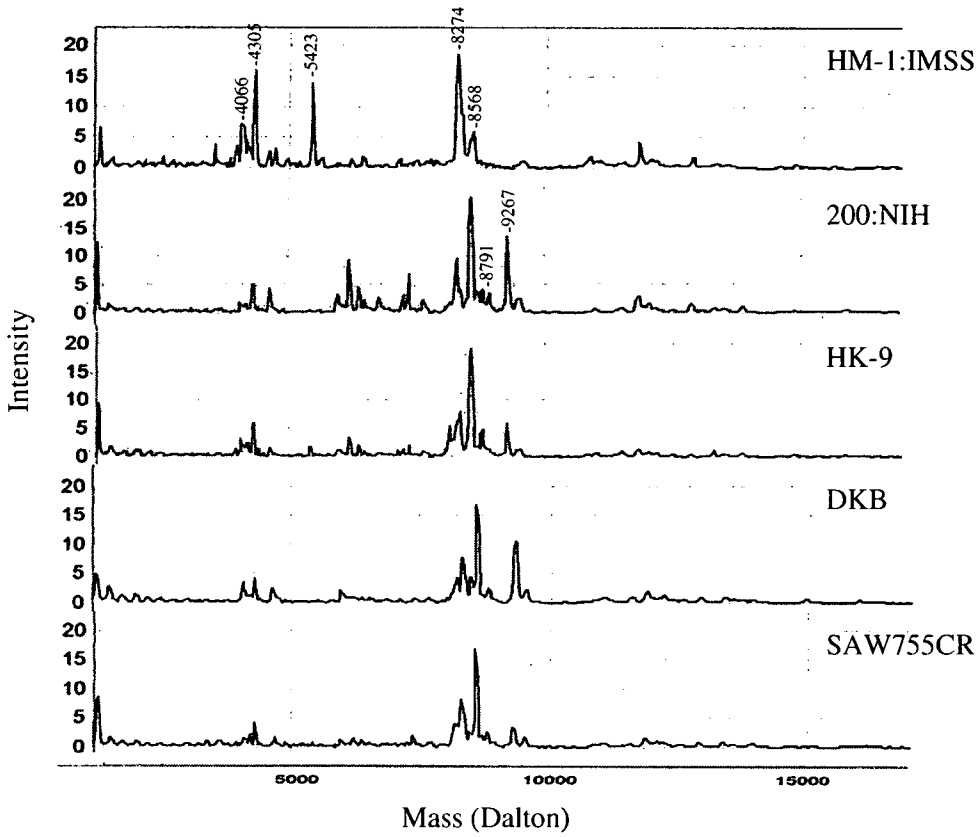

Fig. 2 Representative SELDI-TOF MS spectra of five laboratory strains of *E. histolytica*. The molecular masses are shown above the peaks

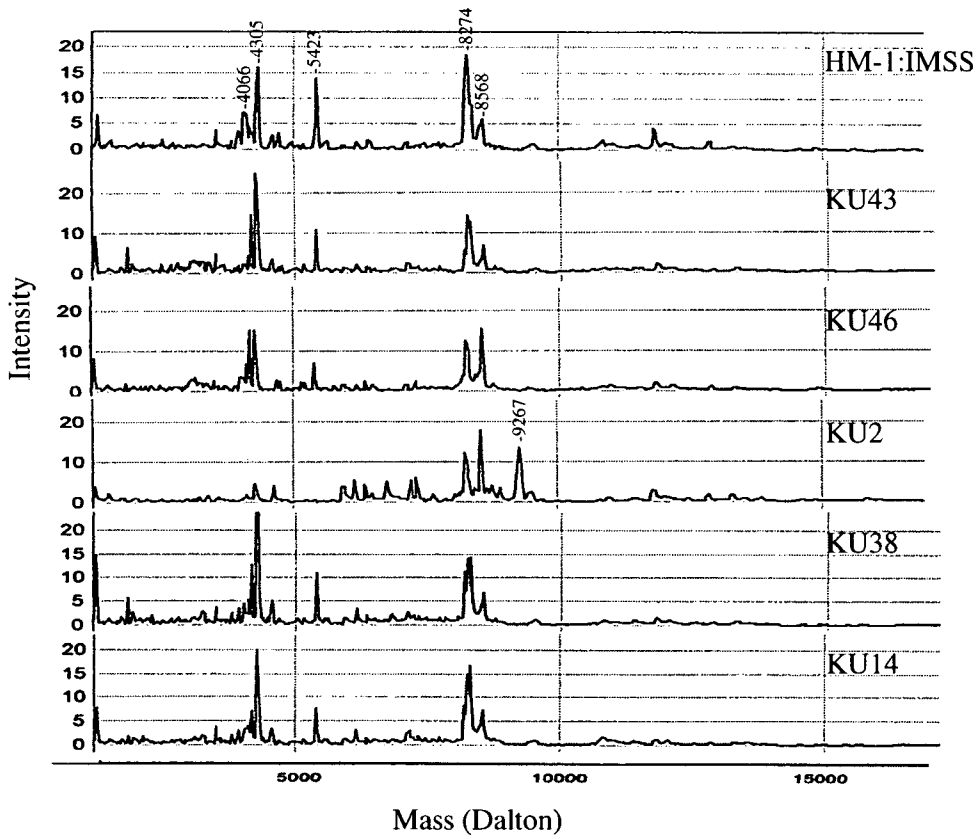

Fig. 3 Representative SELDI-TOF MS spectra of five Japanese isolates of *E. histolytica*. The molecular masses are shown above the major peaks

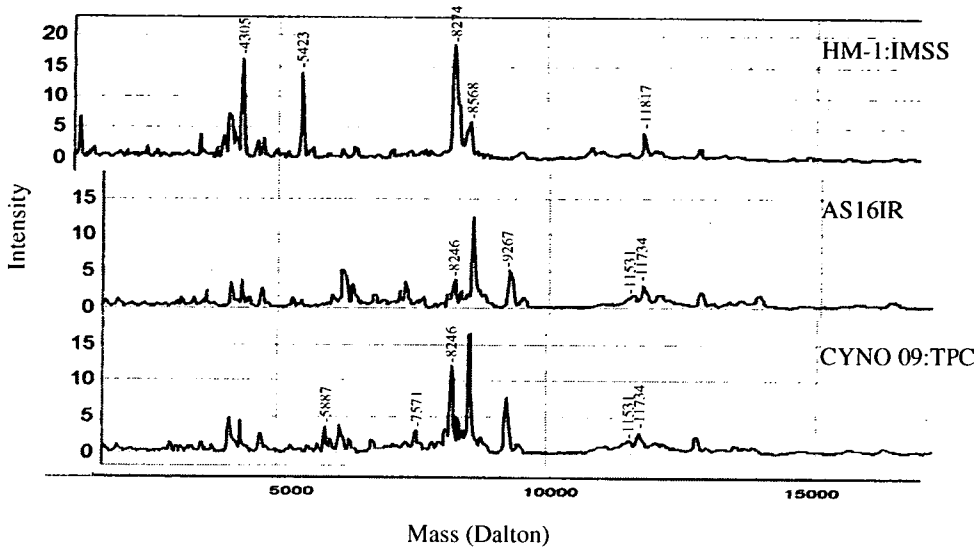

Fig. 4 Representative SELDI-TOF MS spectra of *E. dispar* isolates. The molecular masses are shown above the major peaks

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	CYN009: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		
CYN009:TPC	29.2	53.8	58.5	72.3	63.1	44.6	46.2	43.1	47.7	55.4	81.5	

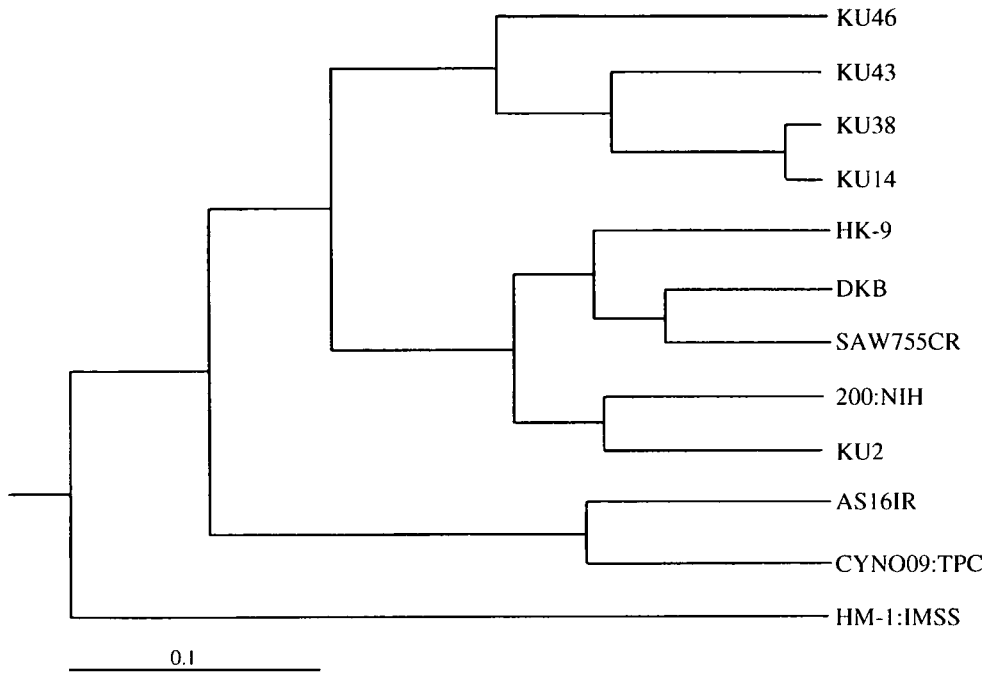

Fig. 6 Dendrogram representing relations among *E. histolytica* and *E. dispar* isolates by the UPGMA method

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.

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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

2003, 217 in 2004, 282 in 2005, and 277 in 2006) (Table 1). All individuals provided informed consent. Outpatient age was the only additional information. The anti-EH antibody-positive sera were examined for anti-*Chlamydia trachomatis* (CT) and anti-TP antibodies as indicators of STDs.

ELISA: EH antigens were prepared from axenically cultured EH (HM-1: ATCC No. 50527). The antigen was diluted with 0.05 M bicarbonate buffer to yield a concentration of 5 μg/mL. The diluted antigen (100 μL) was pipetted into each well of the microplate (Nunc-Immuno Module; Nunc Co., Roskilde, Denmark; Cat. No. 469078) and sensitized by incubation for 2 h at 37°C (7). After washing with a buffer (0.15 M phosphate buffer (PB) containing 0.05% Tween 20, pH 7.2; PB/T), 100 μL of the serum samples diluted 1:200 with a dilution buffer (PB/T containing 1% bovine serum albumin, BSA) were pipetted into the microwells followed by incubation for 40 min at 37 °C. The microplate was washed 3 times with PB/T after incubation, and 100 μL of 1:8000 diluted peroxidase-conjugated anti-human IgG rabbit serum (ICN-Cappel Inc., Aurora, OH, USA; Cat. No. 55221) was added, followed by incubation for 40 min at 37°C. After washing with PB/T, the substrate solution comprising 0.03% 2,2′-azino-bis(3-ethylbenzo thiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma Co., Cat. No. A1888), 0.01% H_2O_2 in 10 mL of 0.1 M Na_2HPO_4, and 10 mL of 0.1 M citric acid was added to each well. After 7 min, 50 μL of 1.25% NaF solution was added to arrest color development, and an ELISA reader (MTP-120) (Corona Electric Co. Ltd., Ibaragi, Japan) was used to measure the absorbance at 405 nm. The cut-off S/N optical density (OD) value, calculated using the average OD of negative sera from 5 healthy individuals, was set at 3.

Serological test for CT and TP infections: Anti-CT IgG and IgA antibodies were measured using a solid-phase enzyme immunoassay kit (Peptide-Chlamydia IgG and

IgA; Ani Labsystems Ltd., Oy, Vantaa, Finland).

Nontreponemal anti cardiolipin (CL) antibodies were detected using 2 kits (biologic false-positive tests for syphilis), i.e., slide test antigen (DS Pharma-Biomedicals, Osaka, Japan) and rapid plasma reagin (RPR) test (Sanko Junyaku, Tokyo, Japan). The sera that tested positive by these kits were retested by using the TP passive hemagglutination (TPHA) kit (Fujirebio Inc., Tokyo, Japan) for detection of anti-TP antibody. The OD values of the positive and negative control sera for quality control and the OD value of the positive control of the kits were measured during each test run of all the serological tests.

ELISA with the antigens from the virulence-augmented amoebae: Based on the hypothesis that the amount of antigenic substances also decreased simultaneously with loss of virulence, we attempted to design an ELISA with better sensitivity in the following manner: 1) HM-1 and LA526 strains cultured axenically for 3 days in the TYI-S-33 medium were inoculated (dose, 1×10^6 amoebae/0.1 mL/head) into the left hepatic lobes of female Syrian golden hamsters (age, 3–4 weeks) (8). 2) On 6th day of inoculation, the hamsters were sacrificed and the livers dissected aseptically, the amoebic abscesses isolated from each of the livers were minced fine and crushed using scissors for medical use in the TYI-S-33 medium (9). 3) After removing the tissue debris from the amoebic cell suspensions and washing twice in the TYI-S-33 medium by centrifugation ($175 \times g$ for 3 min), the 2 EH strains were cultured axenically in the TYI-S-33 medium. They were named LHM-1 and LLA526. The long term axenically cultured HM-1 was passaged 16 times through hamster liver due to significant reduction in the virulence, whereas the LA526 was passaged only once because it was newly isolated from the pus of a human amoebic liver abscess only 8 months ago. 4) LHM-1 and LLA526 were mass cultured within 2 weeks after transferring into

TYI-S-33 medium from the amoebic liver abscesses and they were harvested and washed twice in phosphate buffered saline (PBS) by centrifugation (175 × *g* for 3 min), and suspended in 5 mL of distilled water, followed by intermittent sonication (UH-150; SMT Co. Ltd, Tokyo, Japan) at 10 kHz for 5 min in an ice bath. 5) The sonicated suspensions were then centrifuged at 9,100 × *g* for 30 min, and the protein concentrations of the aqueous soluble extracts were measured by Bradford's method (10). 6) LHM-1 and LLA526 antigens were sensitized at a concentration of 0.5 μg/well according to the procedures described above.

Each serum sample was tested in triplicate for each of the 3 antigens, i.e., LHM-1, LLA526, and HM-1, and the average OD values were calculated. The sensitivity of ELISA for each of the 3 antigens was compared with the positive serum samples of 5 patients clinically diagnosed to have amoebic liver abscesses and 5 mentally handicapped persons in a rehabilitation institution for the intellectually impaired in Japan who were almost free from symptoms of amoebiasis but were positive for EH cysts on microscopy and for EH antigen when tested by using an EH-specific antigen detection kit (*E. histolytica* II kit; TechLab, Blacksburg, Va, USA). In each of the 10 human serum samples obtained as described above from the cases of amoebic liver abscess and asymptomatic cyst passers, the ratio between the S/N values (OD value of serum sample (S)/average OD of negative sera from 5 healthy individuals (N)) of ELISA with the LHM-1 and HM-1 antigens and that between the S/N values of ELISA with the LLA526 and HM-1 antigens was determined.

RESULTS

Seroprevalence of anti-EH antibodies in the female population: During the 4 years, i.e., from 2003 to 2006, in the 981 sera obtained from the study population, the

seroprevalence of anti-EH (HM-1) antibodies increased every year. In 2005 and 2006, the annual positive rate was >5%; the average of the annual positive rates over the 4 years was 4.1% (40/981) (Table 2). In addition, 60%, i.e., 24/40 of these cases were also positive for anti-CT antibodies—an indicator of STDs. On the contrary, none of the cases were positive for anti-CL antibodies (retest by TPHA kit was not performed). The strong positive correlation between seropositivity for anti-EH and anti-CT antibodies suggested sexual transmission of EH in the female population. In terms of age, the highest number of individuals positive for anti-EH antibodies was observed in 11 individuals aged 25–29 years and in 9 individuals aged 30–34 years. The number of individuals positive for anti-CT antibodies correlated with the number of those positive for anti-EH antibodies (Table 3).

Comparison of S/N values of ELISA with 3 different antigens: The results of a pilot study in which the ELISA with LHM-1 and LLA526 antigens was conducted using sera from 5 human cases each of amoebic liver abscess and asymptomatic cyst passers are shown in Figure 1. The average ratio (1.324) between S/N values of ELISA with the LHM-1 and HM-1 antigens and that (1.254) between S/N values of ELISA with the LLA526 and HM-1 antigens increased significantly in only asymptomatic cases with low S/N values ($p < 0.05$ by *t*-test) and not in ALA cases (1.048 and 1.006, respectively; $p > 0.05$ by *t*-test).

The 40 anti-HM-1 antibody-positive sera as detected by ELISA were classified into 2 groups based on the magnitude of the S/N values (i.e., groups I and II with S/N values ≥10 and <10, respectively). The tendency of ELISA with LHM-1 and LLA526 antigens to yield significantly higher S/N values ($p < 0.01$ by *t*-test) was also confirmed in seropositive cases from among the present study population with low S/N values (<10) by ELISA using the HM-1 antigen (Figure 2).

DISCUSSION

In Japan, the MSM population is still thought to be a major high-risk group for STDs. However, our study provided evidence indicating that the seroprevalence of the EH infection in the female population of Tokyo is increasing annually.

In addition, the result that 60% of the female study population who were anti-EH antibody-positive were also positive for anti-CT antibodies, an indicator of STD, along with the diversity of sexual behavior suggested that a major proportion of females positive for anti-EH antibodies were possibly infected with EH by sexual transmission. We do not fully understand why none of the cases were positive for anti-CL antibodies in the female population unlike the MSM population (11, 12). We are, currently, conducting further epidemiological studies on the route of EH infection in the female population.

The tendency of ELISA using the LHM-1 and LLA526 antigens to yield statistically higher S/N values ($p < 0.01$ by *t*-test) was evident only in the positive cases with low S/N values (<10) among the present female study population. The active antigenic substance that brought about this effect could not be identified in the present study. Despite the necessity for further evaluation, the improved ELISA is expected to be an effective method for detecting anti-EH antibodies from such asymptomatic patients who have low antibody titers, and the hamster liver-passaged EH may be applied as a sensitive antigen to other serodiagnostic methods, such as dot-ELISA (13) and immunofluorescence antibody (IFA) test (14).

The control of amoebiasis should start with efforts toward raising the awareness of the general public regarding the risk of infection by sexual transmission because of their indifference to STD, and establishing more sensitive and simple mass

examination methods, such as the newly designed ELISA using the antigen extracted from the virulence-augmented EH strains, that have a better sensitivity for the diagnosis of amoebiasis.

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Figure legends

Figure 1. Ratio of the S/N values of ELISA with better sensitivity performed using the antigens from hamster liver-passaged LHM-1 and LLA526 strains to the S/N values of ELISA using the antigen from the HM-1:IMSScl6 (HM-1) strain in the clinical serum samples of amoebiasis (S/N values: OD of serum sample (S)/average OD of negative sera (N)].

The mean of the ratios of triplicate ELISA are plotted. I: Samples (n = 5); anti-HM-1 antibody-positive sera from the clinical patients of amoebic liver abscess. The S/N values of ELISA with LHM-1 and LLA526 antigens did not increase significantly ($p > 0.05$ by the *t*-test). II: Samples (n = 5); anti-HM-1 antibody-positive sera from the mentally handicapped individuals admitted to a rehabilitation institution for the intellectually impaired in Japan. The S/N values of ELISA with LHM-1 and LLA526 antigens increased significantly ($p < 0.05$ by the *t*-test).

Figure 2. Ratio of S/N values of ELISA performed using antigens from the LHM-1 and LLA526 strains to the S/N values of conventional ELISA performed using the antigen from the HM-1 strain in female outpatients from a gynecologist's office. The mean of the ratios of triplicate ELISA are plotted.

I: Samples (n = 16); anti-HM-1 antibody-positive sera with high S/N values (≥10). The S/N values of ELISA with LHM-1 and LLA526 antigens did not increase significantly ($p > 0.05$ by the *t*-test). II: Samples (n = 24); anti-HM-1 antibody-positive sera with low S/N values (<10). The S/N values by ELISA with LHM-1 and LLA526 antigens increased significantly ($p < 0.01$ by *t*-test).

Table 1. Study samples in age categories from 2003 to 2006

Age	2003	2004	2005	2006	Total
< 20	15	18	22	12	67
20–24	53	56	71	79	259
25–29	69	59	83	90	301
30–34	41	54	57	41	193
35–39	10	18	25	24	77
40–44	8	4	8	12	32
45–49	2	1	8	1	12
50 <	1	2	5	7	15
unknown	6	5	3	11	25
	205	217	282	277	981

Table 2. Seroprevalence of anti-*Entamoeba histolytica* antibodies in the female outpatients from a gynecologist's office, Tokyo, Japan, by enzyme-linked immunosorbent assay (ELISA) from 2003 to 2006

Year	No. of samples	No. of positives	Positive rate %	No. of positives for anti-CT antibodies[1)]
2003	205	3	1.5	2
2004	217	8	3.7	6
2005	282	14	5.0	7
2006	277	15	5.4	9
Total	981	40	4.1	24

1): Number of positives for anti-*Chlamyidia trachomatis* (CT) antibodies that were also positive for anti-*Entamoeba histolytica* antibodies.

Table 3. Age distribution of the female outpatients from a gynecologist's office with positive for anti-*E. histolytica* antibodies

Year	20–24	25–29	30–34	35–39	40–44	45–49	50 <
2003	1	1 (1)		1 (1)			
2004		2 (1)	3 (3)	2 (2)			
2005	1	2 (1)	3 (1)		3	4 (3)	2 (2)
2006	3 (2)	6 (5)	3 (2)		2		1
Total	5 (2)	11 (8)	9 (6)	3 (3)	5	4 (3)	3 (2)

Number of positives for anti-*Chlamyidia trachomatis* (CT) antibodies that were also positive for anti-*Entamoeba histolytica* antibodies are provided in parentheses