

Original Article

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

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SUMMARY: From 2003 to 2006, we surveyed the seroprevalence of amoebic infection in female outpatients at a gynecologist's office, which was 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 increased during that period, and anti-*Chlamydia trachomatis* antibodies were detected in 60%, i.e., 24 of 40 anti-HM-1 antibody-positive individuals, suggesting sexual transmission of *E. histolytica*. We designed an ELISA with better sensitivity using the antigen extracted from the virulence-augmented *E. histolytica* 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 were significantly higher in intestinal amoebiasis cases with low S/N values than in amoebic liver abscess cases. In the present study of the 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. This modification of the antigen preparation for ELISA is expected to be effective in detecting anti-*E. histolytica* antibodies from such asymptomatic patients who have low antibody titers.

INTRODUCTION

In Japan, it was thought until the mid-1970s that amoebiasis was solely food borne and spread via food contaminated with cysts of *Entamoeba histolytica*. However, in the late 1970s, after amoebiasis was reported to have spread among men having sex with men (MSM) in large cities of the United States, it was recognized as a sexually transmitted disease (STD) (1,2). Within a few years, the suspected number of MSM having anti-*E. histolytica* antibodies along with anti-*Treponema pallidum* antibodies began to increase in densely populated cities in Japan (3,4).

In data provided by Japan's National Epidemiological Surveillance of Infectious Diseases, the number of notified cases with amoebiasis has been increasing annually; in 2006, 747 cases were reported, approximately 90% of which 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-*E. histolytica* (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 *E. histolytica* strains by liver-passaging in hamsters. The serum anti-*E. histolytica* 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 Tokyo gynecologist's office that was designated as an STD sentinel clinic by the Tokyo Metropolitan Government. We collected blood samples from 981 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. Patient age was the only additional information. The anti-*E. histolytica* antibody-positive sera were examined for anti-*Chlamydia trachomatis* and anti-*T. pallidum* antibodies as indicators of STDs.

ELISA: *E. histolytica* antigens were prepared from axeni-

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
Total	205	217	282	277	981

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cally cultured *E. histolytica* (HM-1; ATCC no. 50527). The antigen was diluted with 0.05 M bicarbonate buffer to yield a concentration of 5 $\mu\text{g}/\text{mL}$. 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 1:8,000 diluted peroxidase-conjugated anti-human IgG rabbit serum (ICN-Cappel Inc., Aurora, Ohio, 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., St. Louis, Mo., USA; 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 *C. trachomatis* and *T. pallidum* infections: Anti-*C. trachomatis* 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 two 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 using the *T. pallidum* passive hemagglutination (TPHA) kit (Fujirebio, Inc., Tokyo, Japan) for detection of anti-*T. pallidum* 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 run of all the serological tests.

ELISA with antigens from virulence-augmented amoebae: Based on the hypothesis that the amount of antigenic substances would also decrease simultaneously with the loss of virulence, we attempted to design an ELISA with better sensitivity in the following manner: (i) 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). (ii) On the 6th day of inoculation, the hamsters were sacrificed and the livers dissected aseptically. The amoebic abscesses isolated from each of the livers were minced finely and crushed using scissors for medical use in the TYI-S-33 medium (9). (iii) 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), both *E. histolytica* 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 the significant reduction in 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 earlier. (iv) LHM-1 and LLA526

were mass cultured within 2 weeks after their transfer into TYI-S-33 medium from the amoebic liver abscesses. The antigens were then harvested and washed twice in phosphate buffered saline (PBS) by centrifugation ($175 \times g$ 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. (v) The sonicated suspensions were then centrifuged at $9,100 \times g$ for 30 min, and the protein concentrations of the aqueous soluble extracts were measured by Bradford's method (10). (vi) LHM-1 and LLA526 antigens were sensitized at a concentration of 0.5 $\mu\text{g}/\text{well}$ according to the procedures described above.

Each serum sample was tested in triplicate for each of the three antigens—LHM-1, LLA526, and HM-1—and the average OD values were calculated. The sensitivity of ELISA for each of the three antigens was compared with the positive serum samples of 5 patients clinically diagnosed having amoebic liver abscesses and 5 mentally handicapped persons in a rehabilitation institution for the intellectually impaired in Japan, who were almost free from amoebiasis symptoms but positive for *E. histolytica* cysts on microscopy and for *E. histolytica* antigen when tested by using an *E. histolytica*-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 was determined 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.

RESULTS

Seroprevalence of anti-*E. histolytica* antibodies in the female population: During the 4 years 2003 to 2006, in the 981 sera samples obtained from the study population, the seroprevalence of anti-*E. histolytica* (HM-1) antibodies increased every year. In 2005 and 2006, the annual positive rate was >5%; the average annual positive rate 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-*C. trachomatis* antibodies—an indicator of STDs. On the other hand, none of the cases were positive for anti-CL antibodies (a retest by the TPHA kit was not performed). The strong positive correlation between seropositivity for anti-*E. histolytica* and anti-*C. trachomatis* antibodies suggested sexual transmission of *E. histolytica* in the female population. The age range with the highest number of individuals positive for anti-*E. histolytica* antibodies was that of 25-29 years, with 11, and that of 30-

Table 2. Seroprevalence of anti-*Entamoeba histolytica* antibodies in the female outpatients from a gynecologist's office, Tokyo, Japan, by enzyme-linked immunosorbent assay 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-*Chlamydia trachomatis* (CT) antibodies that were also positive for anti-*E. histolytica* antibodies.

Table 3. Age distribution of the female outpatients from a gynecologist's office with positive for anti-*Entamoeba 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-*Chlamydia trachomatis* antibodies that were also positive for anti-*E. histolytica* antibodies are provided in parentheses.

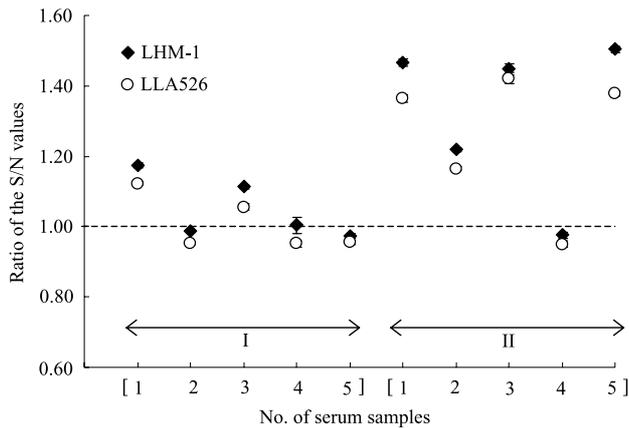


Fig. 1. Ratio of the S/N values of ELISA with better sensitivity performed using the antigens from hamster liver-passaged LHM-1 and LLA526 strains to the S/N values of ELISA using the antigen from the HM-1:IMSSc6 (HM-1) strain in the clinical serum samples of amoebiasis (S/N values: OD of serum sample [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 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 t test).

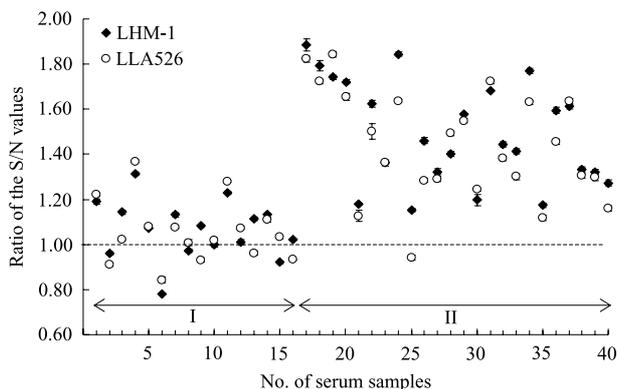


Fig. 2. Ratio of S/N values of ELISA performed using antigens from the LHM-1 and LLA526 strains to the S/N values of conventional ELISA performed using the antigen from the HM-1 strain in female outpatients from a gynecologist's office. The mean of the ratios of triplicate ELISA are plotted. I: Samples ($n = 16$); 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 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).

34 years, with 9. The number of individuals positive for anti-*C. tracomatis* antibodies correlated with the number of those positive for anti-*E. histolytica* antibodies (Table 3).

Comparison of S/N values of ELISA with three different antigens: Figure 1 shows the results of a pilot study in which ELISA with LHM-1 and LLA526 antigens was conducted using sera from 5 human cases each of amoebic liver abscess and asymptomatic cyst passers. 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 only in asymptomatic cases with low S/N values ($P < 0.05$ by t test) and not in amoebic liver abscess 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 two 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 *E. histolytica* infection in the female population of Tokyo is increasing annually.

In addition, the result that 60% of the female study population who were anti-*E. histolytica* antibody-positive were also positive for anti-*C. tracomatis* antibodies, an indicator of STD, along with the diversity of sexual behavior suggested that a major proportion of females positive for anti-*E. histolytica* antibodies may have been infected with *E. histolytica* 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 case in the MSM population (11,12). We are currently conducting further epidemiological studies on the route of *E. histolytica* 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 of further evaluation, the improved ELISA is expected to be effective for detecting anti-*E. histolytica* antibodies from such asymptomatic patients who have low antibody titers. Moreover, the hamster liver-passaged *E. histolytica* may be applied as a sensitive antigen to other serodiagnostic methods, such as dot-ELISA (13) and immunofluorescence antibody tests (14).

Because of the public's indifference to STDs, the control of amoebiasis should start with efforts to raise public awareness of the risk of infection by sexual transmission. Also simpler and more sensitive mass examination methods should be developed, such as the newly designed ELISA using the antigen extracted from the virulence-augmented *E. histolytica* strains, which have a better sensitivity for the diagnosis of amoebiasis.

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