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Higher seroprevalence of *Entamoeba histolytica* than that of HIV-1 at a voluntary counselling and testing centre in Tokyo

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5 2 **Title:**

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7 3 **Higher seroprevalence of *Entamoeba histolytica* than that of HIV-1 at a voluntary**
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9 4 **counselling and testing centre in Tokyo**

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36 **Keywords:** Parasitology, Epidemiology, Tropical medicine, Diagnostic microbiology, Public
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38 health, Sexual medicine.
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42 **Footnotes:** Preliminary results from this study were presented at ASM Microbe 2018, convened
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44 June 7-11, 2018, in Atlanta, GA, USA.
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3 **43 Abstract (249 words)**
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5 **44 Background** Amebiasis, which is caused by *Entamoeba histolytica*, is a re-emerging public
6 health issue owing to sexually transmitted infection (STI) in Japan. However, epidemiological
7 data are quite limited.
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12 **47 Methods** To reveal the relative prevalence of sexually transmitted *E. histolytica* infection to
13 other STIs, we conducted a cross-sectional study at a voluntary counselling and testing (VCT)
14 centre in Tokyo. Seroprevalence of *E. histolytica* was assessed according to positivity with an
15 enzyme-linked immunosorbent assay for *E. histolytica*-specific IgG in serum samples collected
16 from anonymous VCT clients.
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24 **52 Results** Among 2,083 samples, seropositive rate for *E. histolytica* was 2.64%, which was higher
25 than that for HIV-1 (0.34%, $p < 0.001$) and comparable to that for syphilis (rapid plasma reagin
26 (RPR) 2.11%, $p = 0.31$). Positivity for *Chlamydia trachomatis* in urine by transcription-mediated
27 amplification (TMA) was 4.59%. Seropositivity for *E. histolytica* was high among RPR- or
28 *Treponema pallidum* hemagglutination (TPHA)-positive individuals and it was not different
29 between clients with and without other STIs. Both seropositivity of *E. histolytica* and RPR were
30 high among male clients. The seropositive rate for anti-*E. histolytica* antibody was positively
31 correlated with age. TMA positivity for urine *C. trachomatis* was high among female clients and
32 negatively correlated with age. Regression analysis identified that male sex, older age, and
33 TPHA-positive results are independent risk factors of *E. histolytica* seropositivity.
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62 **Conclusion** Seroprevalence of *E. histolytica* was 7.9 times higher than that of HIV-1 at a VCT
63 centre in Tokyo, with a tendency to be higher among people at risk for syphilis infection.
64

65 **Strengths and limitations of this study**

- 66 ➤ This study is the first examining the seroprevalence of *E. histolytica* at a voluntary
67 counselling and testing (VCT) centre in Tokyo, because of the lack of active surveillance for
68 *E. histolytica* infection, including asymptotically infected individuals.
- 69 ➤ Our findings provided epidemiological evidences that the seropositive rate for *E. histolytica*
70 was significantly 7.9 times higher than that of HIV-1, and was comparable to that for active
71 syphilis infection. It was strongly associated with male sex, older age, and TPHA-
72 positive result.
- 73 ➤ This study design was a cross-sectional study of anonymous clients at a VCT centre. We
74 could not assess risk behaviour or sexual behaviour, and exclude the possibility of selection
75 bias of clients. Further studies are needed to evaluate these factors.
- 76 ➤ To assess seroprevalence of *E. histolytica* in general population, more appropriate sampling
77 locations should be identified, such as STI clinics that are visited by female commercial sex
78 workers. This study population predominantly consisted of male (70.8%).

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3 81 **Text (2,727 words)**
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6 82 **INTRODUCTION**
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8 83 Amebiasis is an enteric protozoa infection caused by *Entamoeba histolytica*. Up to 80% of
9
10 84 *E. histolytica* infections are asymptomatic but persistent; the remainder result in the development
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12 85 of invasive diseases, such as colitis and liver abscess [1]. Asymptomatically infected individuals
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14 86 represent a risk to the community because they are a source of new infections. Transmission
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16 87 occurs via the oral–faecal route. It has long been believed that amebiasis is only endemic in
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18 88 developing countries where food and water are frequently contaminated with human faeces, or
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20 89 that it occurs among travellers to or immigrants from these countries [1, 2]. However, in the
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22 90 previous two decades, it has been reported that cases of amebiasis have been rapidly increasing
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24 91 and have become a re-emerging infectious disease not only in developed countries of East Asia
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26 92 but also in European developed countries [3-12]. Human-to-human transmission occurs via
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28 93 direct sexual contact, such as oral–anal sexual contact and contact among men who have sex
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30 94 with men in these countries [13, 14]. Under such circumstances, it is essential to identify
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32 95 individuals who are asymptomatic but chronically infected with *E. histolytica* and who thus
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34 96 represent sources of new infection, for the epidemiologic control of sexually transmitted *E.*
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36 97 *histolytica* infection. However, little epidemiological data is currently available in Japan, other
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38 98 than that from National Epidemiological Surveillance of Infectious Diseases (NESID), which
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40 99 only reports clinically diagnosed “symptomatic” cases. Moreover, it is critical to understand the
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42 100 epidemiology of sexually transmitted *E. histolytica* infection before the upcoming Tokyo
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44 101 Olympics in 2020, which could serve as a source of the rapid spread of such neglected
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46 102 communicable diseases.
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3 103 In the present study, we investigated the seroprevalence of *E. histolytica* at a voluntary
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5 104 counselling and testing (VCT) centre in Tokyo, in comparison with the prevalence of other
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7 105 sexually transmitted infections (STIs). In addition, we discuss future strategies for the
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10 106 epidemiologic control of sexually transmitted *E. histolytica* infection.

11 12 107 **METHODS**

13 14 15 108 **Setting**

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17 109 Tokyo, the capital city of Japan, is located on the Pacific on the eastern coast of Honshu,
18
19 110 the largest of the four main islands comprising Japan. According to the national surveillance
20
21 111 system, the annual number of HIV tests performed and the incidence rates of HIV infection are
22
23 112 higher in Tokyo than those of other prefectures [15]. The Tokyo Metropolitan Minami Shinjuku
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25 113 Testing – Counselling Centre is the largest HIV testing centre in Tokyo, and it is very close to a
26
27 114 town in Shinjuku with a large population of men who have sex with men (MSM) [16]. Because
28
29 115 there are more MSM who visit this centre to undergo testing for HIV and other STIs, the
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31 116 incidence rate of HIV infection at this centre is higher than that of other public health centres in
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36 117 Tokyo [17].

37 38 118 **Study population, samples, and ethics issues**

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40 119 The design of this study was a cross-sectional study. The total 2,083 serum samples used in
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42 120 this study were collected at the Tokyo Metropolitan Minami Shinjuku Testing – Counselling
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44 121 Centre where more than 10,000 anonymous clients seek HIV-1 screening tests each year.
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46 122 Collected samples are transferred to the Tokyo Metropolitan Institute of Public Health for
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48 123 laboratory testing, then stored at 4°C. Fourth generation HIV-1 screening is performed routinely
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50 124 throughout the year. However, in 2 months of the year (e.g., June and December in the case of
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3 125 2017), the Tokyo Metropolitan Government intensifies STI screening, and rapid plasma reagin
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5 126 (RPR) and *Treponema pallidum* hemagglutination (TPHA) tests for syphilis screening are
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7 127 additionally performed for all clients. In addition, urinary sampling and transcription-mediated
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9 128 amplification (TMA) assay testing for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are
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11 129 performed for clients who are willing to undergo these tests. Therefore, we assessed the
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13 130 seroprevalence of anti-*E. histolytica* antibody using stored serum samples collected in June and
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15 131 December of 2017 and compared this with the positivity for other STIs in the present study. In
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17 132 the present study, there was no selection bias or missing data.

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19 133 This study was approved by the ethics committee of the National Center for Global Health
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21 134 and Medicine (NCGM-2302) and Tokyo Metropolitan Institute of Public Health (29-875). All
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23 135 protocols for this study were conducted in accordance with the Declaration of Helsinki.

24 136 **Laboratory testing**

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26 137 The presence of anti-*E. histolytica* antibody was detected using a commercially available
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28 138 ELISA kit (*Entamoeba histolytica* IgG-ELISA; GenWay Biotech, Inc., San Diego, CA. USA).
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30 139 All procedures were performed according to the manufacturer's instructions. In brief, diluted
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32 140 serum samples (100X dilution in IgG sample diluent) as well as 5 control samples, consisting of
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34 141 1 substrate blank, 1 negative control, 2 cut-off controls, and 1 positive control, were applied to
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36 142 96-well plates pre-treated with *E. histolytica* antigen and incubated at 37°C for 1 hour. After
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38 143 washing the plates using washing solution, 100 µL of *E. histolytica* Protein A conjugate was
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40 144 added to all wells except the substrate blank and incubated for 30 minutes in the dark. After a
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42 145 second wash, TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution was added to all wells.

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3 146 After a 15-minute incubation, 100 μ L of stop solution was applied to the plates, and absorbance
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5 147 of the specimen was then read at 450/620 nm using a spectrometer.
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8 148 **Statistical analysis**

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10 149 Of the total samples tested in each STI screening test, the proportion of seropositive blood
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12 150 and urine samples are presented with 95% confidence interval (CIs) calculated using the Wilson–
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14 151 Brown method. The seroprevalence of *E. histolytica* was compared with that of other sexually
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16 152 transmitted infections using Fisher’s exact test. To determine the trend of seropositivity among
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18 153 age groups, we used the chi-square test for trend. Statistical significance was defined as a two-
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20 154 sided p value < 0.05. All statistical analyses were conducted using GraphPad Prism (GraphPad
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22 155 Software, La Jolla, CA, USA). Logistic regression analysis for identification of factors
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24 156 influencing *E. histolytica* seropositivity was performed using Stata (StataCorp LLC., College
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26 157 Station, TX, USA).
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30 158 **Patient and public involvement**

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33 159 Patients and public were not involved in the design and conduct of this research.
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35 160 **RESULTS**

36 161 **Study population and seroprevalence of *E. histolytica* at a voluntary counselling and testing** 37 38 162 **centre in Tokyo**

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41 163 In total, 2,083 samples were analysed. The average age of clients was 35.2 (95% CI: 34.8–
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43 164 35.7) years, and 70.8% (1474/2083) were male (Fig 1). The overall seropositive rate for *E.*
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45 165 *histolytica* was 2.64%; this was significantly higher than that for HIV-1 (0.34%) and the
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47 166 comparable level as that for syphilis by RPR (2.11%) (Fig 2A). The positive rate of urinary TMA
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50 167 for *C. trachomatis* (4.59%) was higher than that for *E. histolytica*; however, urinary TMA testing
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3 168 for *C. trachomatis* and *N. gonorrhoeae* was only carried out in 69.0% (1,437/2,083) of clients,
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5 169 i.e., those who were willing to undergo TMA testing. These results suggest that *E. histolytica* is a
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7 170 more common STI than HIV-1 in Tokyo and is at a level comparable to that of syphilis infection.
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10 171 Interestingly, all individuals who were seropositive for *E. histolytica* were seronegative for HIV-
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12 172 1 (Fig 2B). Furthermore, the seropositive rate for *E. histolytica* was significantly higher among
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14 173 people who were seropositive for syphilis infection (by both RPR and TPHA) than among those
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16 174 who were seronegative for syphilis; no significant differences in *E. histolytica* seropositivity
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18 175 were seen according to TMA positivity for *C. trachomatis*. These results indicate that *E.*
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20 176 *histolytica* infection is spreading among people at risk for syphilis infection.
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24 177 **Differences in seropositivity by sex and age group**

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26 178 Next, we compared positivity for STIs between male and female clients. The seropositive
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28 179 rate for *E. histolytica* was significantly higher in male (3.46%) than in female (0.66%) clients, as
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30 180 seen for syphilis infection (RPR: 2.78% vs. 0.49% and TPHA: 9.29% vs. 0.82%) (Fig 3A). The
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32 181 proportion of urinary TMA results positive for *C. trachomatis* was significantly higher in female
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34 182 (8.77%) than male (2.65%) clients. However, it is difficult to simply compare the TMA
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36 183 positivity by sex because persistent, asymptomatic *C. trachomatis* infection of the urinary tract
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38 184 occurs more frequently in females [18-21]. Moreover, the age of female clients was significantly
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40 185 lower than that of males, and the proportion of clients aged 29 years or less in females was
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42 186 53.4% whereas that in males was only 29.6% (Fig 1). These results indicate that both male and
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44 187 female clients in this study are at risk for STIs; however, the predominant pathogens might differ
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46 188 between relatively older males (*E. histolytica* and *T. pallidum*) and relatively younger females
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48 189 (*C. trachomatis*).
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3 190 To determine the trend of *E. histolytica* seropositivity by age, we compared seropositivity
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5 191 for *E. histolytica* in different age groups. Interestingly, the seropositive rates for anti-*E.*
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7 192 *histolytica* antibody and RPR was highest among clients aged 50 years or older (5.41% and
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9 193 2.70%, respectively). Moreover, a positive correlation was observed between age and
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11 194 seropositivity for *E. histolytica* (Fig 3B). Positive urinary TMA for *C. trachomatis* was highest
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13 195 among clients aged 29 years or younger (8.35%) and showed a negative correlation with age.
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15 196 These results are consistent with national surveillance data, in which diagnosed cases of
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17 197 *Chlamydia* infection have a peak in the 20s [22], whereas the median age of reported cases of
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19 198 amebiasis is relatively high (50 years in men and 40 years in women) [5, 20]. Considering these
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21 199 findings, *E. histolytica* infection might be more prevalent among relatively older age groups (40
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23 200 years or more) whereas *Chlamydia* infection is more prevalent in relatively younger populations.

201 **Risk of seropositivity for *E. histolytica***

202 Finally, to identify the risk factors of seropositivity for *E. histolytica*, we performed
203 logistic regression analysis using data of client characteristics and the results of STI screening
204 tests. Univariate and multivariate regression analyses revealed that male sex, a history of syphilis
205 infection (by TPHA), and older age were independent risk factors of seropositivity for *E.*
206 *histolytica* (Table 1). In particular, age 40 years or older was a high-risk factor of seropositivity
207 for *E. histolytica* (odds ratio 3.31 in people aged less than 40 years, p value < 0.001 by univariate
208 analysis; data not shown). In addition, univariate analysis showed that positive RPR was a high-
209 risk factor for *E. histolytica* seropositivity; however, this was diminished in multivariate analysis
210 owing to the strong association with TPHA positivity. Univariate analysis using preliminary
211 urinary TMA data of 1,437 participants showed that positivity for *C. trachomatis* in the urine had
212 no impact on *E. histolytica* seropositivity (Table 1). We could not include HIV-1 serology and

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3 213 TMA positivity for *N. gonorrhoeae* in urine in the logistic regression analyses because no clients
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5 214 who were HIV-1 seropositive or positive for *N. gonorrhoeae* by TMA were also seropositive for
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8 215 *E. histolytica*.
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216
217 **Table 1. Impact of individual characteristics of seropositivity for *Entamoeba histolytica*,**
218 **Tokyo.**

| | | Univariate analysis | | Multivariate analysis*** | |
|--|------------------------------------------------|----------------------|---------|--------------------------|---------|
| | | OR | p value | OR | p value |
| | | (95% CI) | | (95% CI) | |
| | Sex (Male) | 5.42 (1.95–15.06) | < 0.001 | 3.17 (1.10–9.07) | 0.032 |
| | Older age (by 10-year age groups) | 1.66 (1.33–2.08) | < 0.001 | 1.49 (1.17–1.90) | 0.001 |
| | HIV-1 positive | ND** | | | |
| | Syphilis infection | | | | |
| | RPR positive | 5.1 (1.93–13.49) | 0.006 | 1.26 (0.41–3.89) | 0.693 |
| | TPHA positive | 6.19 (3.37–11.39) | < 0.001 | 4.30 (2.11–8.76) | < 0.001 |
| | Urine <i>C. trachomatis</i> (TMA) positive* | 1.93 (0.58–6.47) | 0.326 | | |
| | Urine <i>N. gonorrhoeae</i> (TMA) positive* | ND** | | | |

219 * Data of urinary TMA testing available only for 69.0% (1,437 of 2,083) of total clients.

220 ** Odds ratios could not be determined in logistic regression analysis because all clients who
221 were HIV-1 positive and/or positive for gonorrhoea by TMA were *E. histolytica* seronegative.

222 *** Multivariate analysis for age and sex, plus factors with $p < 0.05$ in univariate analysis.

223 Abbreviations: OR, odds ratio; RPR, rapid plasma reagin; TPHA, *Treponema pallidum*
224 hemagglutination; TMA, transcription-mediated amplification; ND, not determined.

225

226 **DISCUSSION**

227 The most important finding of the present study was that the seroprevalence of *E.*
228 *histolytica* was significantly (7.9 times) higher than that of HIV-1 and it was comparable to that
229 for syphilis (by RPR). Certainly, it is difficult to simply compare seropositivity among these
230 three tests; the HIV-1 screening test continues to be positive for a person's entire life whereas
231 positivity in RPR and anti-*E. histolytica* antibody tests indicate current or recent infection [21,
232 22]. However, these results strongly indicate that the endemicity of *E. histolytica* in Tokyo is
233 higher than that of HIV-1 and close to the level of syphilis. In contrast to our seroprevalence
234 data, the national surveillance data of Japan from NESID pragmatically show that the annual
235 number of diagnosed cases of amebiasis (1,151 in 2016) is not only much lower than that of
236 syphilis (4,575 cases), it is also lower than that of HIV-1 (1,443 cases) [22, 23]. Our results
237 suggest that the endemicity of amebiasis in Japan is currently underestimated, thereby remaining
238 a neglected disease in Japan despite frequently reported life-threatening cases of amebiasis [26-
239 29]. Interestingly, in the present study, all individuals who were seropositive for *E. histolytica*
240 were HIV-1 negative whereas regression analysis identified that seropositivity for syphilis by
241 TPHA was an independent risk factor of a positive result for anti-*E. histolytica* antibody.
242 Previous reports have emphasized the high seroprevalence of *E. histolytica* [30] and increasing
243 number of amebiasis cases [31-33] among individuals with HIV-1 infection. Although the
244 epidemiological trend of *E. histolytica* among HIV-1-positive individuals could not be assessed
245 in this study owing to the small number of clients who were positive for HIV-1, it should be
246 noted that sexually transmitted *E. histolytica* infection is currently spreading even among HIV-1-

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3 247 negative populations, as we indicated in our previous hospital-based cross-sectional analysis
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5 248 [34]. Currently, screening for *E. histolytica* is not routinely performed at VCT centres in Japan;
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8 249 however, public health interventions should be considered to control sexually transmitted *E.*
9
10 250 *histolytica* infection.

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12 251 The clinical significance of seropositivity for *E. histolytica* remains unclear and is beyond
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14 252 the scope of this paper. Serologic testing is a sensitive diagnostic method for symptomatic
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17 253 invasive amebiasis; however, positive results are also obtained for recent infections, up to the
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19 254 previous several years [27]. However, we previously reported that 70.4% of *E. histolytica*-
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21 255 seropositive individuals did not have any amebiasis-related symptoms nor any history of
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23 256 treatment for amebiasis. Interestingly, 20% of such individuals in a Japanese HIV-1 cohort
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26 257 developed symptomatic invasive amebiasis within a 1-year follow-up period [27]. In another
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28 258 cross-sectional analysis, we also reported that ulcerative lesions owing to *E. histolytica* in the
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30 259 large intestine are frequently identified (7/18, 38.9%) by colonoscopy among asymptomatic
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32 260 individuals who are *E. histolytica* seropositive whereas these rarely (1/53, 1.9%) occur among *E.*
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34 261 *histolytica*-seronegative people [35]. Serologic screening for *E. histolytica* at VCT centres,
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36 262 followed by diagnosis of subclinical *E. histolytica* infection by colonoscopy and treatment at a
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38 263 referral hospital, is one possible public health strategy against sexually transmitted *E. histolytica*
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40 264 infection. However, we must assess the utility of serologic testing for the screening of
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42 265 asymptomatic *E. histolytica* in well-designed prospective analyses in the future.

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44
45 266 The present study has some limitations that should be considered. First, this preliminary
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47 267 investigation was a cross-sectional study of anonymous clients at a VCT centre. We could not
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49 268 assess risk behaviour or sexual behaviour with respect to seropositivity for *E. histolytica* owing
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51 269 to a lack of detailed data on the characteristics of clients. In addition, the study periods were 2

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3 270 months apart owing to the availability of data for not only HIV-1 but also other STIs (serum tests
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5 271 for syphilis and urine tests for chlamydia and gonorrhoea). We could not exclude the possibility
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7 272 of selection bias of clients, such as those who undergo repeat testing. Second, anti-*E. histolytica*
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9 273 antibody was screened using stored serum. Long periods of storage could lead to lower
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11 274 sensitivity of serologic tests, resulting in underestimation of the seroprevalence of *E. histolytica*.
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13 275 Third, we obtained a considerably lower seropositive rate for *E. histolytica* among female clients
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15 276 (0.66%, 4/609) than that among males (3.46%, 51/1,474). This probably results from the fact that
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17 277 VCT centres may not be appropriate for identifying female populations at high risk for *E.*
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19 278 *histolytica* infection; our female clients were relatively younger and had lower seropositive rates
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21 279 in RPR and TPHA tests. More appropriate sampling locations should be identified, such as STI
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23 280 clinics that are visited by female commercial sex workers [36].

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28 281 In conclusion, among clients of a VCT centre in Tokyo, seropositivity for *E. histolytica*
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30 282 was 7.9 times higher than that of HIV-1 and tended to be high among individuals at risk of
31
32 283 syphilis infection. Active detection and treatment of asymptomatic cases of *E. histolytica*
33
34 284 infection should be considered for the epidemiologic control of sexually transmitted *E.*
35
36 285 *histolytica* infection in Japan.

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287

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291

292 **Contributors**

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298 **Competing Interests**

299 None.

301 **Patient Consent**

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309 **Data sharing statement**

310 No additional data available.

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404 Figure legends

405 **Figure 1. Proportion of clients in each age group among men and women.** The average age
406 among female clients was significantly lower than that in male clients ($p < 0.001$). The
407 proportion of clients aged 29 years or less among female clients was 53.4% whereas that in male
408 clients was only 29.6%.

409 **Figure 2. Seropositivity for *Entamoeba histolytica* and other sexually transmitted infections**
410 **(STIs) in Tokyo.** Serologic testing results (anti-*E. histolytica* antibody, HIV-1, RPR, and TPHA)
411 were obtained for 2,083 clients of a voluntary counselling and testing centre in June and
412 December of 2017. Results of urinary TMA for *Chlamydia trachomatis* and *Neisseria*
413 *gonorrhoeae* were available for 1,437 clients who agreed to testing. All statistics were calculated
414 using Fisher's exact test. (A) The seropositive rate for *E. histolytica* was compared with those of
415 other STIs. (B) Comparison of seropositivity for *E. histolytica*, with and without other STIs.
416 Abbreviations: CI, confidence interval; RPR, rapid plasma reagin; TPHA, *Treponema pallidum*
417 hemagglutination; TMA, transcription-mediated amplification.

418 **Figure 3. Positive rate of sexually transmitted infections (STIs) by sex and age group.** (A)
419 Positive rate of *Entamoeba histolytica* and other STIs were compared between male ($n = 1474$)
420 and female ($n = 609$) clients using Fisher's exact test. (B) Seropositive rates for *E. histolytica* and
421 RPR, and TMA positivity for *Chlamydia trachomatis* were calculated for clients of different age
422 groups (serum, urine samples): 29 years or younger (752, 503), 30–39 years (666, 453), 40–49
423 years (443, 315), and 50s or older (222, 167). Correlation between age and positivity was
424 calculated using the chi-square test for trend. Abbreviations: RPR, rapid plasma reagin test;
425 TPHA, *Treponema pallidum* hemagglutination; TMA, transcription-mediated amplification.

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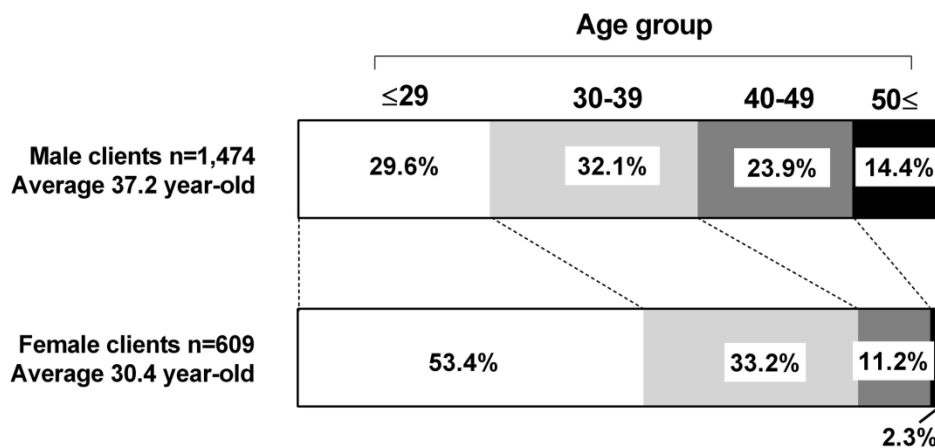


Figure 1. Proportion of clients in each age group among men and women. The average age among female clients was significantly lower than that in male clients ($p < 0.001$). The proportion of clients aged 29 years or less among female clients was 53.4% whereas that in male clients was only 29.6%.

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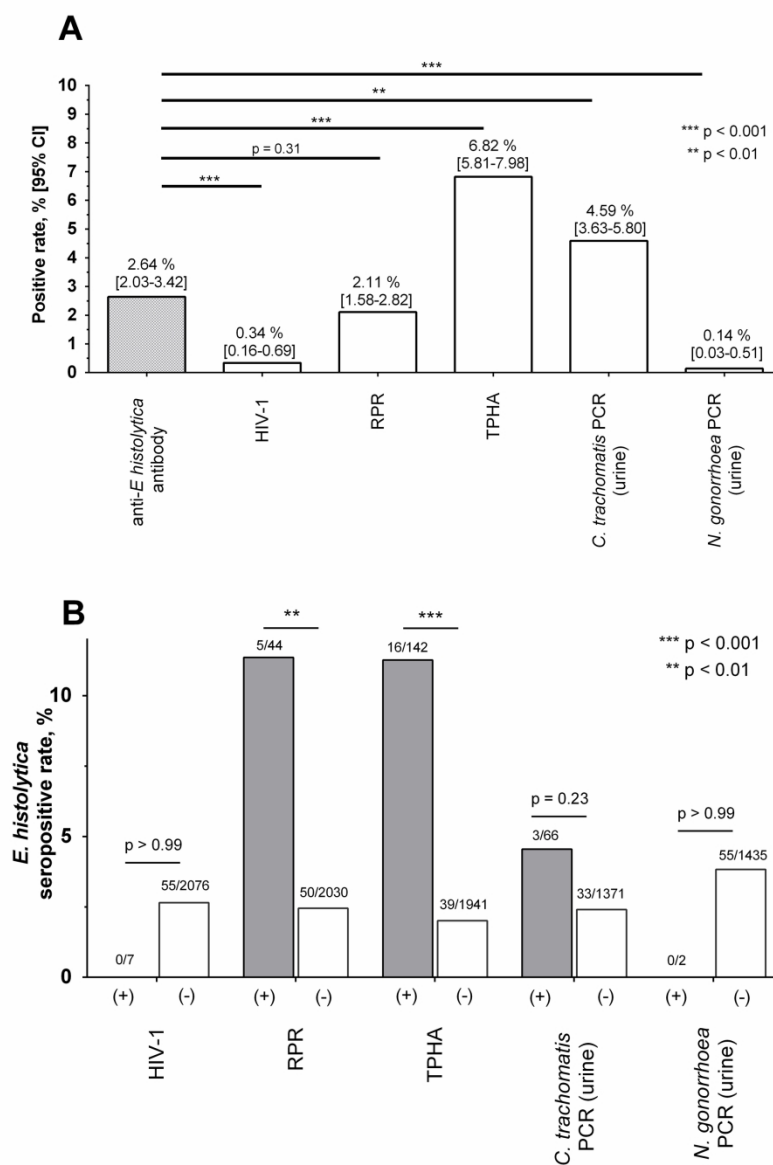


Figure 2. Seropositivity for *Entamoeba histolytica* and other sexually transmitted infections (STIs) in Tokyo. Serologic testing results (anti-*E. histolytica* antibody, HIV-1, RPR, and TPHA) were obtained for 2,083 clients of a voluntary counselling and testing centre in June and December of 2017. Results of urinary TMA for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* were available for 1,437 clients who agreed to testing. All statistics were calculated using Fisher's exact test. (A) The seropositive rate for *E. histolytica* was compared with those of other STIs. (B) Comparison of seropositivity for *E. histolytica*, with and without other STIs. Abbreviations: CI, confidence interval; RPR, rapid plasma reagin; TPHA, *Treponema pallidum* hemagglutination; TMA, transcription-mediated amplification.

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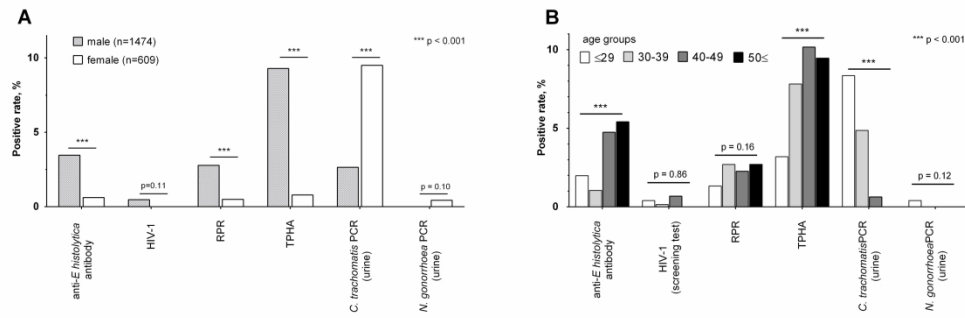


Figure 3. Positive rate of sexually transmitted infections (STIs) by sex and age group. (A) Positive rate of *Entamoeba histolytica* and other STIs were compared between male (n = 1474) and female (n = 609) clients using Fisher's exact test. (B) Seropositive rates for *E. histolytica* and RPR, and TMA positivity for *Chlamydia trachomatis* were calculated for clients of different age groups (serum, urine samples): 29 years or younger (752, 503), 30–39 years (666, 453), 40–49 years (443, 315), and 50s or older (222, 167). Correlation between age and positivity was calculated using the chi-square test for trend. Abbreviations: RPR, rapid plasma reagin test; TPHA, *treponema pallidum* hemagglutination; TMA, transcription-mediated amplification.

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STROBE Statement—Checklist of items that should be included in reports of *cross-sectional studies*

| | Item No | Recommendation |
|------------------------------|---------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Title and abstract | 1 | (a) Indicate the study's design with a commonly used term in the title or the abstract [Indicated in the method section of the abstract on page 3] (b) Provide in the abstract an informative and balanced summary of what was done and what was found [Provided in method and results section of abstract on page 3] |
| Introduction | | |
| Background/rationale | 2 | Explain the scientific background and rationale for the investigation being reported [Explained in the introduction section of manuscript on page 4] |
| Objectives | 3 | State specific objectives, including any prespecified hypotheses [Stated at the end of the introduction part of manuscript on page 5] |
| Methods | | |
| Study design | 4 | Present key elements of study design early in the paper [Presented under methods section of the manuscript on page 5] |
| Setting | 5 | Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection [Described under methods section of the manuscript on page 5] |
| Participants | 6 | (a) Give the eligibility criteria, and the sources and methods of selection of participants [Given under methods section on page 5-6] |
| Variables | 7 | Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable [Defined under methods section on pages 6, diagnostic details provided in serological testing under methods page 5-6] |
| Data sources/ measurement | 8* | For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group [Described under participants and statistical analysis section of methods, pages 5-6] |
| Bias | 9 | Describe any efforts to address potential sources of bias [Described under study population, samples, and ethics issues section of methods, page 5-6] |
| Study size | 10 | Explain how the study size was arrived at [Explained in sample size under methods, page 5] |
| Quantitative variables | 11 | Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why [Explained in methods, statistics page 6-7] |
| Statistical methods | 12 | (a) Describe all statistical methods, including those used to control for confounding [Described in statistics page 7] (b) Describe any methods used to examine subgroups and interactions [Described in statistics page 5-7] (c) Explain how missing data were addressed [Described in study population, samples, and ethics issues section of methods, page 6] (d) If applicable, describe analytical methods taking account of sampling strategy [N/A] (e) Describe any sensitivity analyses [N/A] |
| Results | | |
| Participants | 13* | (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed [Reported in results section page 7] (b) Give reasons for non-participation at each stage [N/A] (c) Consider use of a flow diagram [N/A] |

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|----|--------------------------|-----|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1 | | | |
| 2 | Descriptive data | 14* | (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders [Given in results section page 7-9] |
| 3 | | | (b) Indicate number of participants with missing data for each variable of interest [N/A] |
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| 6 | Outcome data | 15* | Report numbers of outcome events or summary measures [Reported in results section pages 7-11] |
| 7 | | | |
| 8 | Main results | 16 | (a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included [Described under method (page 5-6) and results and tables pages 7-9] |
| 9 | | | (b) Report category boundaries when continuous variables were categorized [Reported under results, figures, and tables page 8-10] |
| 10 | | | (c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period [N/A] |
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| 18 | Other analyses | 17 | Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses [N/A] |
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| 21 | Discussion | | |
| 22 | Key results | 18 | Summarise key results with reference to study objectives [Page 11] |
| 23 | Limitations | 19 | Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias [Discussed on page 12-13] |
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| 28 | Interpretation | 20 | Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence [Given on page 12-13] |
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| 32 | Generalisability | 21 | Discuss the generalisability (external validity) of the study results [Discussed on page 12-13] |
| 33 | | | |
| 34 | Other information | | |
| 35 | Funding | 22 | Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based [Given in acknowledgement section page 15] |
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*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Seroprevalence of *Entamoeba histolytica* at a voluntary counselling and testing centre in Tokyo: a cross-sectional study

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|---------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
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| Primary Subject Heading: | Epidemiology |
| Secondary Subject Heading: | Infectious diseases, Public health |
| Keywords: | PARASITOLOGY, Epidemiology < INFECTIOUS DISEASES, Tropical medicine < INFECTIOUS DISEASES, Diagnostic microbiology < INFECTIOUS DISEASES, Public health < INFECTIOUS DISEASES, SEXUAL MEDICINE |
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1
2
3 1 **Research article**

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5 2 **Title:**

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7 3 **Seroprevalence of *Entamoeba histolytica* at a voluntary counselling and testing centre in**
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10 4 **Tokyo: a cross-sectional study**

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14 6 Yasuaki Yanagawa ^{1,2#}, Mami Nagashima ^{3#}, Hiroyuki Gatanaga ^{1,4}, Yoshimi Kikuchi ¹, Shinichi
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17 7 Oka ^{1,4}, Keiko Yokoyama ³, Takayuki Shinkai ³, Kenji Sadamasu ³, and Koji Watanabe ^{1,2*}

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38 health, Sexual medicine.
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43 June 7-11, 2018, in Atlanta, GA, USA.
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3 **43 Abstract (266 words)**
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5 **44 Background** Amebiasis, which is caused by *Entamoeba histolytica*, is a re-emerging public
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47 Methods To reveal the relative prevalence of sexually transmitted *E. histolytica* infection to
48 other STIs, we conducted a cross-sectional study at a voluntary counselling and testing (VCT)
49 centre in Tokyo. Seroprevalence of *E. histolytica* was assessed according to positivity with an
50 enzyme-linked immunosorbent assay for *E. histolytica*-specific IgG in serum samples collected
51 from anonymous VCT clients.

52 Results Among 2,083 samples, seropositive rate for *E. histolytica* was 2.64%, which was higher
53 than that for HIV-1 (0.34%, $p < 0.001$) and comparable to that for syphilis (rapid plasma reagin
54 (RPR) 2.11%, $p = 0.31$). Positivity for *Chlamydia trachomatis* in urine by transcription-mediated
55 amplification (TMA) was 4.59%. Seropositivity for *E. histolytica* was high among RPR- or
56 *Treponema pallidum* hemagglutination (TPHA)-positive individuals and it was not different
57 between clients with and without other STIs. Both seropositivity of *E. histolytica* and RPR were
58 high among male clients. The seropositive rate for anti-*E. histolytica* antibody was positively
59 correlated with age. TMA positivity for urine *C. trachomatis* was high among female clients and
60 negatively correlated with age. Regression analysis identified that male sex, older age, and
61 TPHA-positive results are independent risk factors of *E. histolytica* seropositivity.

62 Conclusions Seroprevalence of *E. histolytica* was 7.9 times higher than that of HIV-1 at a VCT
63 centre in Tokyo, with a tendency to be higher among people at risk for syphilis infection. There
64 is a need for education and specific interventions against this parasite, as a potentially re-
65 emerging pathogen.

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3 **66 Strengths and limitations of this study**
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- 5 67 ➤ This study is the first to examine the seroprevalence of *E. histolytica* at a voluntary
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7 68 counselling and testing (VCT) centre in Tokyo, where active surveillance for *E. histolytica*
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9 69 infection is lacking, including among asymptotically infected individuals.
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11 70 ➤ This was a cross-sectional study of anonymous clients at a VCT centre. Thus, comparisons
12
13 71 are possible of the seroprevalence of *E. histolytica* with other STIs.
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15 72 ➤ We could not assess risk behaviour or sexual behaviour and cannot exclude the possibility of
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17 73 selection bias among VCT centre clients.
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3 75 **Text (2,614 words)**
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6 76 **INTRODUCTION**
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8 77 Amebiasis is an enteric protozoa infection caused by *Entamoeba histolytica*. Up to 80% of
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10 78 *E. histolytica* infections are asymptomatic but persistent; the remainder result in the development
11
12 79 of invasive diseases, such as colitis and liver abscess [1]. Asymptomatically infected individuals
13
14 80 represent a risk to the community because they are a source of new infections. Transmission
15
16 81 occurs via the oral–faecal route. It has long been believed that amebiasis is only endemic in
17
18 82 developing countries where food and water are frequently contaminated with human faeces, or
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20 83 that it occurs among travellers to or immigrants from these countries [1, 2]. However, in the
21
22 84 previous two decades, it has been reported that cases of amebiasis have been rapidly increasing
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24 85 and have become a re-emerging infectious disease not only in developed countries of East Asia
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26 86 but also in European developed countries [3-12]. Human-to-human transmission occurs via
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28 87 direct sexual contact, such as oral–anal sexual contact and contact among men who have sex
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30 88 with men in these countries [13, 14]. Under such circumstances, it is essential to identify
31
32 89 individuals who are asymptomatic but chronically infected with *E. histolytica* and who thus
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34 90 represent sources of new infection, for the epidemiologic control of sexually transmitted *E.*
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36 91 *histolytica* infection. However, little epidemiological data is currently available in Japan, other
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38 92 than that from National Epidemiological Surveillance of Infectious Diseases (NESID), which
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40 93 only reports clinically diagnosed “symptomatic” cases. Moreover, it is critical to understand the
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42 94 epidemiology of sexually transmitted *E. histolytica* infection before the upcoming Tokyo
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44 95 Olympics in 2020, which could serve as a source of the rapid spread of such neglected
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46 96 communicable diseases.
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3 97 The aim of the present study was to investigate the seroprevalence of *E. histolytica* at a
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5 98 voluntary counselling and testing (VCT) centre in Tokyo, in comparison with the prevalence of
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8 99 other sexually transmitted infections (STIs). In addition, we discuss future strategies for the
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10 100 epidemiologic control of sexually transmitted *E. histolytica* infection.

11 12 101 **METHODS**

13 14 15 102 **Setting**

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17 103 Tokyo, the capital city of Japan, is located on the Pacific on the eastern coast of Honshu,
18
19 104 the largest of the four main islands comprising Japan. According to the national surveillance
20
21 105 system, the annual number of HIV tests performed and the incidence rates of HIV infection are
22
23 106 higher in Tokyo than those of other prefectures [15]. The Tokyo Metropolitan Minami Shinjuku
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25 107 Testing – Counselling Centre is the largest HIV testing centre in Tokyo, and it is very close to a
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27 108 town in Shinjuku with a large population of men who have sex with men (MSM) [16]. Because
28
29 109 there are more MSM who visit this centre to undergo testing for HIV and other STIs, the
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31 110 incidence rate of HIV infection at this centre is higher than that of other public health centres in
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33 111 Tokyo [17].

34 35 36 112 **Study population, samples, and ethics issues**

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38 113 The design of this study was a cross-sectional study. The total 2,083 serum samples used in
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40 114 this study were collected at the Tokyo Metropolitan Minami Shinjuku Testing – Counselling
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42 115 Centre where more than 10,000 anonymous clients seek HIV-1 screening tests each year.
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44 116 Collected samples are transferred to the Tokyo Metropolitan Institute of Public Health for
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46 117 laboratory testing, then stored at 4°C. Fourth generation HIV-1 screening is performed routinely
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48 118 throughout the year. However, in 2 months of the year (e.g., June and December in the case of
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3 119 2017), the Tokyo Metropolitan Government intensifies STI screening, and rapid plasma reagin
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5 120 (RPR) and *Treponema pallidum* hemagglutination (TPHA) tests for syphilis screening are
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7 121 additionally performed for all clients. In addition, urinary sampling and transcription-mediated
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9 122 amplification (TMA) assay testing for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are
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11 123 performed for clients who are willing to undergo these tests. Therefore, we assessed the
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13 124 seroprevalence of anti-*E. histolytica* antibody using stored serum samples collected in June and
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15 125 December of 2017 and compared this with the positivity for other STIs in the present study. In
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17 126 the present study, there was no selection bias or missing data.

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19 127 This study was approved by the ethics committee of the National Center for Global Health
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21 128 and Medicine (NCGM-2302) and Tokyo Metropolitan Institute of Public Health (29-875). All
22
23 129 protocols for this study were conducted in accordance with the Declaration of Helsinki.

24 130 **Laboratory testing**

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26 131 The presence of anti-*E. histolytica* antibody was detected using a commercially available
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28 132 ELISA kit (*Entamoeba histolytica* IgG-ELISA; GenWay Biotech, Inc., San Diego, CA. USA).
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30 133 All procedures were performed according to the manufacturer's instructions. In brief, diluted
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32 134 serum samples (100X dilution in IgG sample diluent) as well as 5 control samples, consisting of
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34 135 1 substrate blank, 1 negative control, 2 cut-off controls, and 1 positive control, were applied to
35
36 136 96-well plates pre-treated with *E. histolytica* antigen and incubated at 37°C for 1 hour. After
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38 137 washing the plates using washing solution, 100 µL of *E. histolytica* Protein A conjugate was
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40 138 added to all wells except the substrate blank and incubated for 30 minutes in the dark. After a
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42 139 second wash, TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution was added to all wells.

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3 140 After a 15-minute incubation, 100 µL of stop solution was applied to the plates, and absorbance
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5 141 of the specimen was then read at 450/620 nm using a spectrometer.
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8 142 **Statistical analysis**

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10 143 Of the total samples tested in each STI screening test, the proportion of seropositive blood
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12 144 and urine samples are presented with 95% confidence interval (CIs) calculated using the Wilson–
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14 145 Brown method. The seroprevalence of *E. histolytica* was compared with that of other sexually
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16 146 transmitted infections using Fisher’s exact test. To determine the trend of seropositivity among
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18 147 age groups, we used the chi-square test for trend. Statistical significance was defined as a two-
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20 148 sided p value < 0.05. All statistical analyses were conducted using GraphPad Prism (GraphPad
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22 149 Software, La Jolla, CA, USA). Logistic regression analysis for identification of factors
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24 150 influencing *E. histolytica* seropositivity was performed using Stata (StataCorp LLC., College
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26 151 Station, TX, USA).
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30 152 **Patient and public involvement**

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32 153 Patients and public were not involved in the design and conduct of this research.
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35 154 **RESULTS**

36 155 **Study population and seroprevalence of *E. histolytica* at a voluntary counselling and testing** 37 38 156 **centre in Tokyo**

39
40 157 In total, 2,083 samples were analysed. The average age of clients was 35.2 (95% CI: 34.8–
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42 158 35.7) years, and 70.8% (1474/2083) were male (Fig 1). The overall seropositive rate for *E.*
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44 159 *histolytica* was 2.64%; this was significantly higher than that for HIV-1 (0.34%) and the
45
46 160 comparable level as that for syphilis by RPR (2.11%) (Fig 2A). The positive rate of urinary TMA
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48 161 for *C. trachomatis* (4.59%) was higher than that for *E. histolytica*; however, urinary TMA testing
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3 162 for *C. trachomatis* and *N. gonorrhoeae* was only carried out in 69.0% (1,437/2,083) of clients,
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5 163 i.e., those who were willing to undergo TMA testing. These results suggest that *E. histolytica* is a
6
7 164 more common STI than HIV-1 in Tokyo and is at a level comparable to that of syphilis infection.
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10 165 Interestingly, all individuals who were seropositive for *E. histolytica* were seronegative for HIV-
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12 166 1 (Fig 2B). Furthermore, the seropositive rate for *E. histolytica* was significantly higher among
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14 167 people who were seropositive for syphilis infection (by both RPR and TPHA) than among those
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16 168 who were seronegative for syphilis; no significant differences in *E. histolytica* seropositivity
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18 169 were seen according to TMA positivity for *C. trachomatis*. These results indicate that *E.*
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20
21 170 *histolytica* infection is spreading among people at risk for syphilis infection.

171 **Differences in seropositivity by sex and age group**

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173 Next, we compared positivity for STIs between male and female clients. The seropositive
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175 rate for *E. histolytica* was significantly higher in male (3.46%) than in female (0.66%) clients, as
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177 seen for syphilis infection (RPR: 2.78% vs. 0.49% and TPHA: 9.29% vs. 0.82%) (Fig 3A). The
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179 proportion of urinary TMA results positive for *C. trachomatis* was significantly higher in female
180
181 (8.77%) than male (2.65%) clients. However, it is difficult to simply compare the TMA
182
183 positivity by sex because persistent, asymptomatic *C. trachomatis* infection of the urinary tract
occurs more frequently in females [18-21]. Moreover, the age of female clients was significantly
lower than that of males, and the proportion of clients aged 29 years or less in females was
53.4% whereas that in males was only 29.6% (Fig 1). These results indicate that both male and
female clients in this study are at risk for STIs; however, the predominant pathogens might differ
between relatively older males (*E. histolytica* and *T. pallidum*) and relatively younger females
(*C. trachomatis*).

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3 184 To determine the trend of *E. histolytica* seropositivity by age, we compared seropositivity
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5 185 for *E. histolytica* in different age groups. Interestingly, the seropositive rates for anti-*E.*
6
7 186 *histolytica* antibody and RPR was highest among clients aged 50 years or older (5.41% and
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9 187 2.70%, respectively). Moreover, a positive correlation was observed between age and
10
11 188 seropositivity for *E. histolytica* (Fig 3B). Positive urinary TMA for *C. trachomatis* was highest
12
13 189 among clients aged 29 years or younger (8.35%) and showed a negative correlation with age.
14
15 190 These results are consistent with national surveillance data, in which diagnosed cases of
16
17 191 *Chlamydia* infection have a peak in the 20s [22], whereas the median age of reported cases of
18
19 192 amebiasis is relatively high (50 years in men and 40 years in women) [5, 20]. Considering these
20
21 193 findings, *E. histolytica* infection might be more prevalent among relatively older age groups (40
22
23 194 years or more) whereas *Chlamydia* infection is more prevalent in relatively younger populations.

195 **Risk of seropositivity for *E. histolytica***

196 Finally, to identify the risk factors of seropositivity for *E. histolytica*, we compared
197 positivity for STIs between clients who were positive and negative for *E. histolytica* (Table 1).
198 Although there were no statistical differences in the positive rates for HIV-1, *C. trachomatis*, or
199 *N. gonorrhoeae*, the positive rates for any STIs were higher in clients who were positive for *E.*
200 *histolytica* than in *E. histolytica*-negative clients (30.56% vs. 10.49%, $p = 0.0001$). Thus, we
201 performed logistic regression analysis using data of client characteristics and the results of STI
202 screening tests. Univariate and multivariate regression analyses revealed that male sex, a history
203 of syphilis infection (by TPHA), and older age were independent risk factors of seropositivity for
204 *E. histolytica* (Table 2). In particular, age 40 years or older was a high-risk factor of
205 seropositivity for *E. histolytica* (odds ratio 3.31 in people aged less than 40 years, p value <
206 0.001 by univariate analysis; data not shown). In addition, univariate analysis showed that

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3 207 positive RPR was a high-risk factor for *E. histolytica* seropositivity; however, this was
4
5 208 diminished in multivariate analysis owing to the strong association with TPHA positivity.
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7 209 Univariate analysis using preliminary urinary TMA data of 1,437 participants showed that
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10 210 positivity for *C. trachomatis* in the urine had no impact on *E. histolytica* seropositivity (Table 2).
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12 211 We could not include HIV-1 serology and TMA positivity for *N. gonorrhoeae* in urine in the
13
14 212 logistic regression analyses because no clients who were HIV-1 seropositive or positive for *N.*
15
16 213 *gonorrhoeae* by TMA were also seropositive for *E. histolytica*.
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214 **Table 1. Comparison of positive results for other STIs between *E. histolytica* seropositive**
 215 **and seronegative clients.**

| | <i>E. histolytica</i> seropositive | <i>E. histolytica</i> seronegative | p value |
|-----------------------------------|------------------------------------|------------------------------------|----------|
| Male, % (n) | 92.73% (51/55) | 70.17% (1423/2028) | 0.0001 |
| Age, mean (SD) | 41.6 (12.56) | 35.1 (10.4) | < 0.0001 |
| Positive rate, % (n) | | | |
| HIV-1 | 0% (0/55) | 0.35% (7/2028) | > 0.999 |
| RPR | 9.09% (5/55) | 1.92% (39/2028) | 0.005 |
| TPHA | 29.09% (16/55) | 6.21% (126/2028) | < 0.0001 |
| Urine <i>C. trachomatis</i> (TMA) | 8.33% (3/36) | 4.50% (63/1401) | 0.227 |
| Urine <i>N. gonorrhoeae</i> (TMA) | 0% (0/36) | 0.14% (2/1401) | > 0.999 |
| Any of the above STIs | 30.56% (11/36) | 10.49% (147/1401) | 0.0001 |

216 The positive rate of any of the other STIs was calculated only in clients whose blood and urine
 217 were tested.

218 Abbreviations: STIs, sexually transmitted infections; RPR, rapid plasma reagin; TPHA,
 219 *Treponema pallidum* hemagglutination; TMA, transcription-mediated amplification.

220

221 **Table 2. Impact of individual characteristics of seropositivity for *Entamoeba histolytica*,**
 222 **Tokyo.**

223 * Data of urinary TMA testing available only for 69.0% (1,437 of 2,083) of total clients.

| | | Univariate analysis | | Multivariate analysis*** | |
|--|------------------------------------------------|----------------------|---------|--------------------------|---------|
| | | OR | p value | OR | p value |
| | | (95% CI) | | (95% CI) | |
| | Sex (Male) | 5.42 (1.95–15.06) | < 0.001 | 3.17 (1.10–9.07) | 0.032 |
| | Older age (by 10-year age groups) | 1.66 (1.33–2.08) | < 0.001 | 1.49 (1.17–1.90) | 0.001 |
| | HIV-1 positive | ND** | | | |
| | Syphilis infection | | | | |
| | RPR positive | 5.1 (1.93–13.49) | 0.006 | 1.26 (0.41–3.89) | 0.693 |
| | TPHA positive | 6.19 (3.37–11.39) | < 0.001 | 4.30 (2.11–8.76) | < 0.001 |
| | Urine <i>C. trachomatis</i> (TMA) positive* | 1.93 (0.58–6.47) | 0.326 | | |
| | Urine <i>N. gonorrhoeae</i> (TMA) positive* | ND** | | | |

224 ** Odds ratios could not be determined in logistic regression analysis because all clients who
 225 were HIV-1 positive and/or positive for gonorrhoea by TMA were *E. histolytica* seronegative.

226 *** Multivariate analysis for age and sex, plus factors with $p < 0.05$ in univariate analysis.

227 Abbreviations: OR, odds ratio; RPR, rapid plasma reagin; TPHA, *Treponema pallidum*

228 hemagglutination; TMA, transcription-mediated amplification; ND, not determined.

229

230 DISCUSSION

231 The most important finding of the present study was that the seroprevalence of *E.*
232 *histolytica* was significantly (7.9 times) higher than that of HIV-1 and it was comparable to that
233 for syphilis (by RPR), indicating that *E. histolytica* is now a potential re-emerging pathogen in
234 our country. Certainly, it is difficult to simply compare seropositivity among these three tests; the
235 HIV-1 screening test continues to be positive for a person's entire life whereas positivity in RPR
236 and anti-*E. histolytica* antibody tests indicate current or recent infection [21, 22]. However, these
237 results strongly indicate that the endemicity of *E. histolytica* in Tokyo is higher than that of HIV-
238 1 and close to the level of syphilis. In contrast to our seroprevalence data, the national
239 surveillance data of Japan from NESID pragmatically show that the annual number of diagnosed
240 cases of amebiasis (1,151 in 2016) is not only much lower than that of syphilis (4,575 cases), it is
241 also lower than that of HIV-1 (1,443 cases) [22, 23]. Our results suggest that the endemicity of
242 amebiasis in Japan is currently underestimated, thereby remaining a neglected disease in Japan
243 despite frequently reported life-threatening cases of amebiasis [24-27]. Interestingly, in the
244 present study, all individuals who were seropositive for *E. histolytica* were HIV-1 negative
245 whereas regression analysis identified that seropositivity for syphilis by TPHA was an
246 independent risk factor of a positive result for anti-*E. histolytica* antibody. Previous reports have
247 emphasized the high seroprevalence of *E. histolytica* [28] and increasing number of amebiasis
248 cases [29-31] among individuals with HIV-1 infection. Although the epidemiological trend of *E.*

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3 249 *histolytica* among HIV-1-positive individuals could not be assessed in this study owing to the
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5 250 small number of clients who were positive for HIV-1, it should be noted that sexually transmitted
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7 251 *E. histolytica* infection is currently spreading even among HIV-1-negative populations, as we
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9 252 indicated in our previous hospital-based cross-sectional analysis [32]. Currently, screening for *E.*
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11 253 *histolytica* is not routinely performed at VCT centres in Japan; however, public health
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13 254 interventions should be considered to control sexually transmitted *E. histolytica* infection.

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17 255 The clinical significance of seropositivity for *E. histolytica* remains unclear and is beyond
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19 256 the scope of this paper. Serologic testing is a sensitive diagnostic method for symptomatic
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21 257 invasive amebiasis; however, positive results are also obtained for recent infections, up to the
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23 258 previous several years [25]. However, we previously reported that 70.4% of *E. histolytica*-
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25 259 seropositive individuals did not have any amebiasis-related symptoms nor any history of
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27 260 treatment for amebiasis. Interestingly, 20% of such individuals in a Japanese HIV-1 cohort
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29 261 developed symptomatic invasive amebiasis within a 1-year follow-up period [28]. In another
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31 262 cross-sectional analysis, we also reported that ulcerative lesions owing to *E. histolytica* in the
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33 263 large intestine are frequently identified (7/18, 38.9%) by colonoscopy among asymptomatic
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35 264 individuals who are *E. histolytica* seropositive whereas these rarely (1/53, 1.9%) occur among *E.*
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37 265 *histolytica*-seronegative people [33]. Serologic screening for *E. histolytica* at VCT centres,
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39 266 followed by diagnosis of subclinical *E. histolytica* infection by colonoscopy and treatment at a
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41 267 referral hospital, is one possible public health strategy against sexually transmitted *E. histolytica*
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43 268 infection. However, we must assess the utility of serologic testing for the screening of
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45 269 asymptomatic *E. histolytica* in well-designed prospective analyses in the future.

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47 270 The present study has some limitations that should be considered. First, this preliminary
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49 271 investigation was a cross-sectional study of anonymous clients at a VCT centre. We could not

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3 272 assess risk behaviours, including sexual orientation, socioeconomic status, sanitation, and dietary
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5 273 habits, with respect to seropositivity for *E. histolytica* owing to a lack of detailed data on the
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7 274 characteristics of clients. Therefore, further intensive epidemiological studies are needed, to
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10 275 assist in developing future intervention approaches for this re-emerging infectious disease. In
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12 276 addition, the study periods were 2 months apart owing to the availability of data for not only
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14 277 HIV-1 but also other STIs (serum tests for syphilis and urine tests for chlamydia and
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16 278 gonorrhoea). We could not exclude the possibility of selection bias of clients, such as those who
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18 279 undergo repeat testing. Second, anti-*E. histolytica* antibody was screened using stored serum.
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20 280 Long periods of storage could lead to lower sensitivity of serologic tests, resulting in
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22 281 underestimation of the seroprevalence of *E. histolytica*. Third, we obtained a considerably lower
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24 282 seropositive rate for *E. histolytica* among female clients (0.66%, 4/609) than that among males
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26 283 (3.46%, 51/1,474). This probably results from the fact that VCT centres may not be appropriate
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28 284 for identifying female populations at high risk for *E. histolytica* infection; our female clients
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31 285 were relatively younger and had lower seropositive rates in RPR and TPHA tests. More
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33 286 appropriate sampling locations should be identified, such as STI clinics that are visited by female
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35 287 commercial sex workers [34].

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38 288 In conclusion, among clients of a VCT centre in Tokyo, seropositivity for *E. histolytica*
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40 289 was 7.9 times higher than that of HIV-1 and tended to be high among individuals at risk of
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42 290 syphilis infection. Active detection and treatment of asymptomatic cases of *E. histolytica*
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44 291 infection should be considered for the epidemiologic control of sexually transmitted *E.*
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46 292 *histolytica* infection in Japan.

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298

299 Contributors

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301 MN, KY, TS, KS. Data analysis and interpretation: YY, MN, KY, TS, KS, KW. Drafting the
302 article: YY, MN, KW. Critical revision of the article: YY, KW. Final approval of the version to
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304

305 Competing Interests

306 None.

307

308 Patient Consent

309 Not acquired.

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314 Data sharing statement

315 Extra data can be accessed via the Dryad data repository at <http://datadryad.org/> with the doi:
316 doi:10.5061/dryad.kd51c5b2h

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3 4044 405 **Figure legends**

6
7 406 **Figure 1. Proportion of clients in each age group among men and women.** The average age
8
9 407 among female clients was significantly lower than that in male clients ($p < 0.001$). The
10
11 408 proportion of clients aged 29 years or less among female clients was 53.4% whereas that in male
12
13 409 clients was only 29.6%.

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15
16 410 **Figure 2. Seropositivity for *Entamoeba histolytica* and other sexually transmitted infections**
17
18 411 **(STIs) in Tokyo.** Serologic testing results (anti-*E. histolytica* antibody, HIV-1, RPR, and TPHA)
19
20 412 were obtained for 2,083 clients of a voluntary counselling and testing centre in June and
21
22 413 December of 2017. Results of urinary TMA for *Chlamydia trachomatis* and *Neisseria*
23
24 414 *gonorrhoeae* were available for 1,437 clients who agreed to testing. All statistics were calculated
25
26 415 using Fisher's exact test. (A) The seropositive rate for *E. histolytica* was compared with those of
27
28 416 other STIs. (B) Comparison of seropositivity for *E. histolytica*, with and without other STIs.
29
30 417 Abbreviations: CI, confidence interval; RPR, rapid plasma reagin; TPHA, *Treponema pallidum*
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32 418 hemagglutination; TMA, transcription-mediated amplification.

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35 419 **Figure 3. Positive rate of sexually transmitted infections (STIs) by sex and age group.** (A)
36
37 420 Positive rate of *Entamoeba histolytica* and other STIs were compared between male ($n = 1474$)
38
39 421 and female ($n = 609$) clients using Fisher's exact test. (B) Seropositive rates for *E. histolytica* and
40
41 422 RPR, and TMA positivity for *Chlamydia trachomatis* were calculated for clients of different age
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43 423 groups (serum, urine samples): 29 years or younger (752, 503), 30–39 years (666, 453), 40–49
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45 424 years (443, 315), and 50s or older (222, 167). Correlation between age and positivity was
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47 425 calculated using the chi-square test for trend. Abbreviations: RPR, rapid plasma reagin test;
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49 426 TPHA, *Treponema pallidum* hemagglutination; TMA, transcription-mediated amplification.

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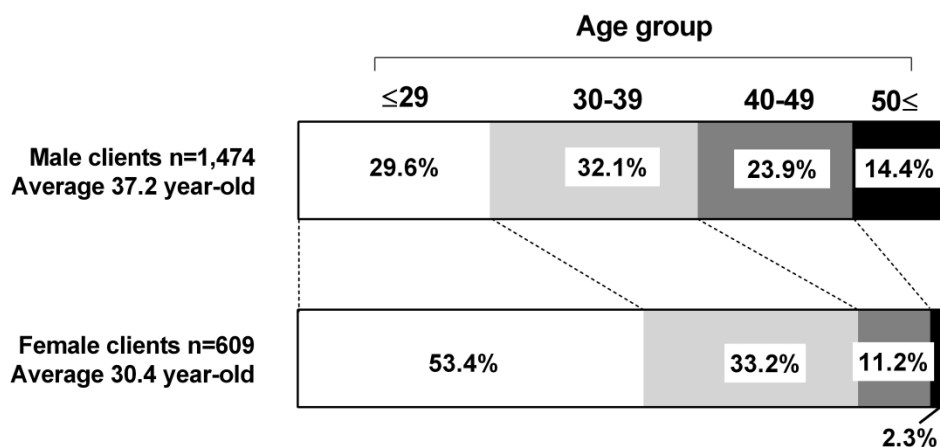


Figure 1. Proportion of clients in each age group among men and women. The average age among female clients was significantly lower than that in male clients ($p < 0.001$). The proportion of clients aged 29 years or less among female clients was 53.4% whereas that in male clients was only 29.6%.

196x175mm (600 x 600 DPI)

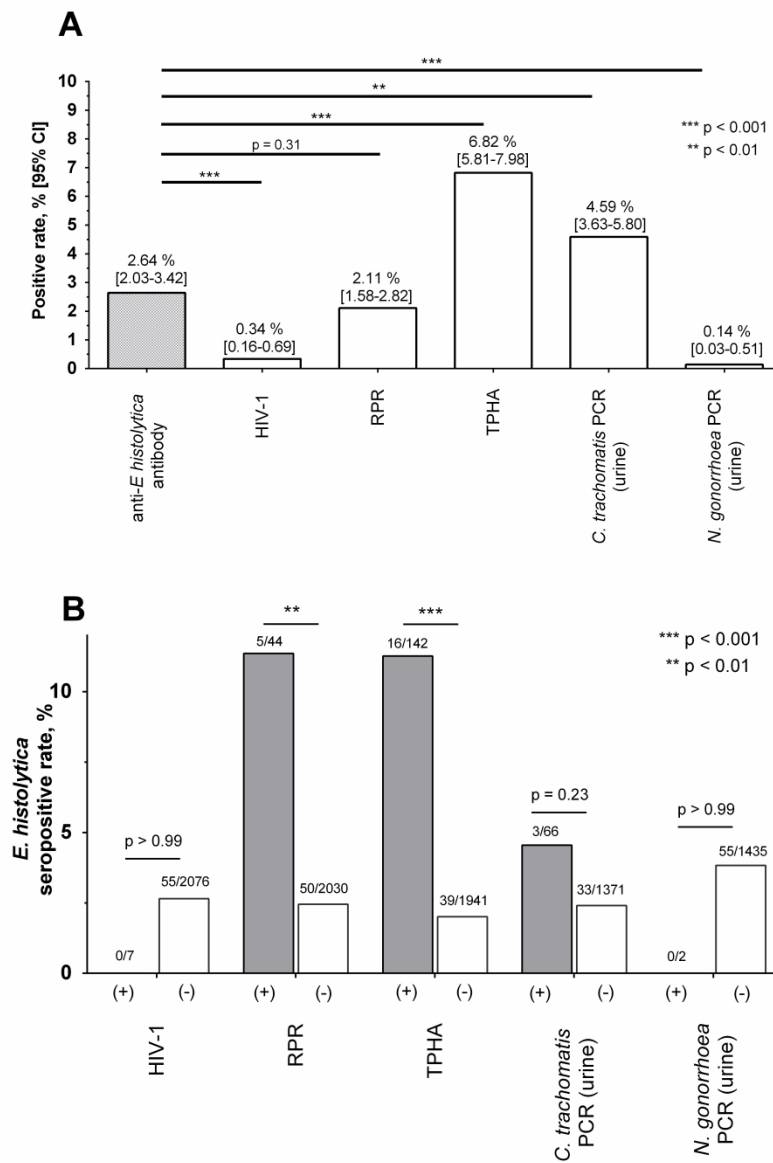


Figure 2. Seropositivity for *Entamoeba histolytica* and other sexually transmitted infections (STIs) in Tokyo. Serologic testing results (anti-*E. histolytica* antibody, HIV-1, RPR, and TPHA) were obtained for 2,083 clients of a voluntary counselling and testing centre in June and December of 2017. Results of urinary TMA for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* were available for 1,437 clients who agreed to testing. All statistics were calculated using Fisher's exact test. (A) The seropositive rate for *E. histolytica* was compared with those of other STIs. (B) Comparison of seropositivity for *E. histolytica*, with and without other STIs. Abbreviations: CI, confidence interval; RPR, rapid plasma reagin; TPHA, *Treponema pallidum* hemagglutination; TMA, transcription-mediated amplification.

190x273mm (600 x 600 DPI)

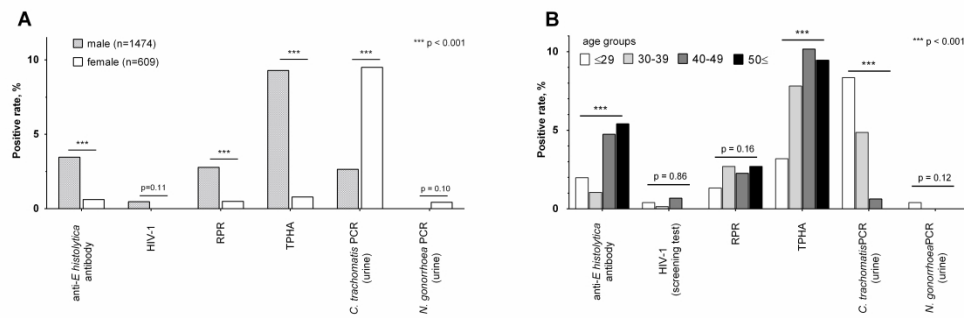


Figure 3. Positive rate of sexually transmitted infections (STIs) by sex and age group. (A) Positive rate of *Entamoeba histolytica* and other STIs were compared between male (n = 1474) and female (n = 609) clients using Fisher's exact test. (B) Seropositive rates for *E. histolytica* and RPR, and TMA positivity for *Chlamydia trachomatis* were calculated for clients of different age groups (serum, urine samples): 29 years or younger (752, 503), 30–39 years (666, 453), 40–49 years (443, 315), and 50s or older (222, 167). Correlation between age and positivity was calculated using the chi-square test for trend. Abbreviations: RPR, rapid plasma reagin test; TPHA, *treponema pallidum* hemagglutination; TMA, transcription-mediated amplification.

278x94mm (600 x 600 DPI)

STROBE Statement—Checklist of items that should be included in reports of *cross-sectional studies*

| | Item No | Recommendation |
|------------------------------|---------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Title and abstract | 1 | (a) Indicate the study's design with a commonly used term in the title or the abstract [Indicated in the method section of the abstract on page 3] (b) Provide in the abstract an informative and balanced summary of what was done and what was found [Provided in method and results section of abstract on page 3] |
| Introduction | | |
| Background/rationale | 2 | Explain the scientific background and rationale for the investigation being reported [Explained in the introduction section of manuscript on page 4] |
| Objectives | 3 | State specific objectives, including any prespecified hypotheses [Stated at the end of the introduction part of manuscript on page 5] |
| Methods | | |
| Study design | 4 | Present key elements of study design early in the paper [Presented under methods section of the manuscript on page 5] |
| Setting | 5 | Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection [Described under methods section of the manuscript on page 5] |
| Participants | 6 | (a) Give the eligibility criteria, and the sources and methods of selection of participants [Given under methods section on page 5-6] |
| Variables | 7 | Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable [Defined under methods section on pages 6, diagnostic details provided in serological testing under methods page 5-6] |
| Data sources/ measurement | 8* | For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group [Described under participants and statistical analysis section of methods, pages 5-6] |
| Bias | 9 | Describe any efforts to address potential sources of bias [Described under study population, samples, and ethics issues section of methods, page 5-6] |
| Study size | 10 | Explain how the study size was arrived at [Explained in sample size under methods, page 5] |
| Quantitative variables | 11 | Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why [Explained in methods, statistics page 6-7] |
| Statistical methods | 12 | (a) Describe all statistical methods, including those used to control for confounding [Described in statistics page 7] (b) Describe any methods used to examine subgroups and interactions [Described in statistics page 5-7] (c) Explain how missing data were addressed [Described in study population, samples, and ethics issues section of methods, page 6] (d) If applicable, describe analytical methods taking account of sampling strategy [N/A] (e) Describe any sensitivity analyses [N/A] |
| Results | | |
| Participants | 13* | (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed [Reported in results section page 7] (b) Give reasons for non-participation at each stage [N/A] (c) Consider use of a flow diagram [N/A] |

| | | | |
|----|--------------------------|-----|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1 | | | |
| 2 | Descriptive data | 14* | (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders [Given in results section page 7-9] |
| 3 | | | (b) Indicate number of participants with missing data for each variable of interest [N/A] |
| 4 | | | |
| 5 | | | |
| 6 | Outcome data | 15* | Report numbers of outcome events or summary measures [Reported in results section pages 7-11] |
| 7 | | | |
| 8 | Main results | 16 | (a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included [Described under method (page 5-6) and results and tables pages 7-9] |
| 9 | | | (b) Report category boundaries when continuous variables were categorized [Reported under results, figures, and tables page 8-10] |
| 10 | | | (c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period [N/A] |
| 11 | | | |
| 12 | | | |
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| 14 | | | |
| 15 | | | |
| 16 | | | |
| 17 | | | |
| 18 | Other analyses | 17 | Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses [N/A] |
| 19 | | | |
| 20 | | | |
| 21 | Discussion | | |
| 22 | Key results | 18 | Summarise key results with reference to study objectives [Page 11] |
| 23 | Limitations | 19 | Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias [Discussed on page 12-13] |
| 24 | | | |
| 25 | | | |
| 26 | | | |
| 27 | | | |
| 28 | Interpretation | 20 | Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence [Given on page 12-13] |
| 29 | | | |
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| 32 | Generalisability | 21 | Discuss the generalisability (external validity) of the study results [Discussed on page 12-13] |
| 33 | | | |
| 34 | Other information | | |
| 35 | Funding | 22 | Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based [Given in acknowledgement section page 15] |
| 36 | | | |
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| 39 | | | |

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.