frequent manifestations of invasive amebiasis are colitis and liver abscess (*1,3–5*). Many persons with *E. histolytica* infection are asymptomatic, but invasive disease develops in 4%–10% of persons with symptomatic *E. histolytica* infections over a 1-year period (*1,6–8*).

Areas with high incidences of amebic infection include India, Africa, Mexico, and parts of Central and South America (*1,2,9*). In countries with low incidence, such as Taiwan, South Korea, and Australia, invasive amebiasis is uncommon, but reports have indicated that amebiasis is an emerging parasitic infection, particularly among men who have sex with men (MSM) (*10–13*). Although epidemics of amebiais have not occurred in Japan, reports from 2001 indicate that invasive amebiasis is common in middle-age men, MSM and HIV-infected patients (*8,14,15*). In Japan, the prevalence of amebiasis has been increasing according to data from the National Epidemiologic Surveillance of Infectious Diseases (*16*). However, the reasons amebiasis is increasing and the actual prevalence of amebic colitis in daily clinical practice have not been fully clarified. Moreover, some studies in Japan have examined risk factors, but most of these studies have reported case series or case reports without control patients (*14,15,17,18*).

Several studies have indicated that HIV infection is a risk for invasive amebiasis, but no consensus has been reached on this issue (*10–12,19*). Furthermore, some researchers have suggested that severe invasive amebiasis may develop in HIV-positive patients (*20–22*). Susceptibility and clinical factors differ between HIV-positive and -negative patients because of differences in immune status. However, the effect of HIV infection on these risk factors for invasive intestinal amebiasis remains unclear.

To address these issues, we clarified annual changes in prevalence and risk factors for amebic colitis among persons who had undergone endoscopy. These factors were then compared between HIV-positive and HIV-negative patients.

Methods

Study Design

We retrospectively reviewed endoscopy records for 14,923 consecutive patients who underwent colonoscopy at the National Center for Global Health and Medicine (NCGM) (Tokyo, Japan) during 2003–2009. Indications for endoscopy included screening for fecal occult blood test; colorectal cancer; anemia; examinations for symptoms such as constipation, loose stool, diarrhea, hematochezia, and abdominal pain; or therapies for colorectal adenoma, early colorectal cancer, and diverticular bleeding.

We excluded patients who had not been tested for HIV infection, syphilis, or hepatitis B virus (HBV) infection. Patients who underwent endoscopic observation only of the anorectal area and those <15 years of age were excluded. A total of 10,930 patients were selected for analysis.

NCGM has 900 beds and is the largest referral center for HIV/AIDS in Japan. Written informed consent for procedures was obtained from all patients before endoscopy and biopsy. The study protocol was approved by the ethics committee of NCGM.

Sexually Transmitted Diseases

We collected laboratory data for sexually transmitted diseases (STDs), such as HIV infection, syphilis, and HBV infection, before endoscopy. Histories of HBV infection and syphilis were defined as presence of antibody against as hepatitis B surface antigen and positive results in a *Treponema pallidum* hemagglutination test, respectively. In Japan, because only health care workers are vaccinated against hepatitis B, positive results for antibody against hepatitis B surface antigen were attributable to vaccination in few cases.

For HIV-positive patients, we determined CD4 cell counts within 1 week of endoscopy. We categorized CD4 cell counts into 4 groups: >300 cells/μL, 201–300 cells/μL, 101–200 cells/μL, and <100 cells/μL Routes of infection were determined by medical staff who questioned each patient at their first visit to the hospital. Routes were classified into 6 categories: homosexual, bisexual, heterosexual, drug use, untreated blood products, and unknown. We defined sexual preference into 2 categories: MSM and heterosexual. Patients who were not homosexual or bisexual were regarded as heterosexual.

Diagnosis of Amebic Colitis Caused by *E. histolytica* Infection

We performed a biopsy and aspirated intestinal fluid from lesions endoscopically when abnormal findings were seen by endoscopy. Amebic colitis was suspected on the basis of endoscopic findings, such as erythema, edematous mucosa, erosions, white exudates, and ulcers (Figure 1) (*22,23*). Negative results for intestinal fluid cultures for bacterial species or acid-fast bacillus were confirmed. Amebic colitis was defined as amebic trophozoites in biopsy specimens stained with both hematoxylin and eosin (Figure 2, panel A) and periodic acid–Schiff (Figure 2, panel B), negative intestinal fluid cultures for other species, negative histologic features for other colonic diseases, and a positive clinical response to metronidazole. Trophozoites showed characteristic hemophagocytosis, which is specific for *E. histolytica* infection (Figure 2, panel A).

Routes of Amebic Infection

When amebic colitis was diagnosed, the physician asked the patient directly for information about the route

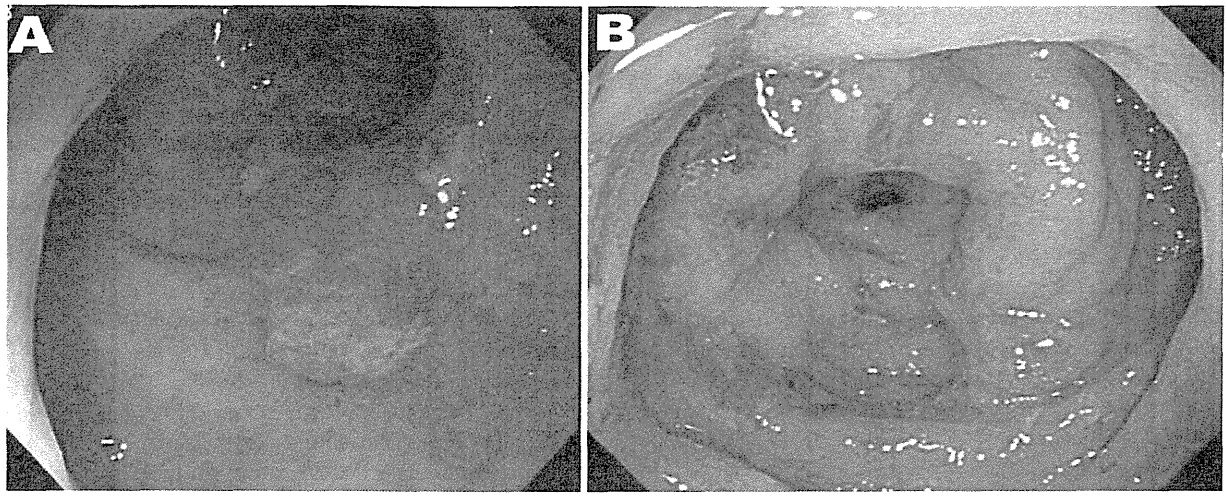

Figure 1. Endoscopic features of amebic colitis, Japan, 2003–2009. A) Colonoscopy showing ulcers in the rectum. B) Colonoscopy showing multiple erosions with exudates surrounded by edematous mucosa in the sigmoid colon.

of amebic infection. The physician confirmed whether the patient had traveled in tropical areas, resided in a facility for the intellectually disabled, was a male or female commercial sex worker (CSW), or had contact with a CSW or MSM. For travel exposure, history of overseas travel in the past year was elicited. Patients to whom none of the above applied were treated as unknown.

Statistical Analysis

We assessed changes in annual prevalence by using the χ^2 test for linear trends. We summarized descriptive data for patients with and without amebic colitis. To determine risk factors for amebic colitis, we estimated the odds ratio (OR) between amebic colitis and clinical factors including age, sex, sexual preference, and history of STDs. We divided patients into 2 age groups, ≥50 years and <50 years. We used a multiple logistic regression model with factors that showed $p<0.2$ by univariate analysis. A final model was then developed by backward selection of factors that showed $p<0.05$. The adequacy of this model was evaluated by using the Hosmer-Lemeshow goodness-of-fit test and a receiver operating characteristic area under the curve.

We also conducted subgroup analysis concerning HIV infection. We investigated interactions between the effect of HIV infection and risk factors for amebic colitis. In HIV-positive patients, the relationship between prevalence of amebic colitis and CD4 cell counts in 4 categories was evaluated by using the χ^2 test for linear trends. All statistical

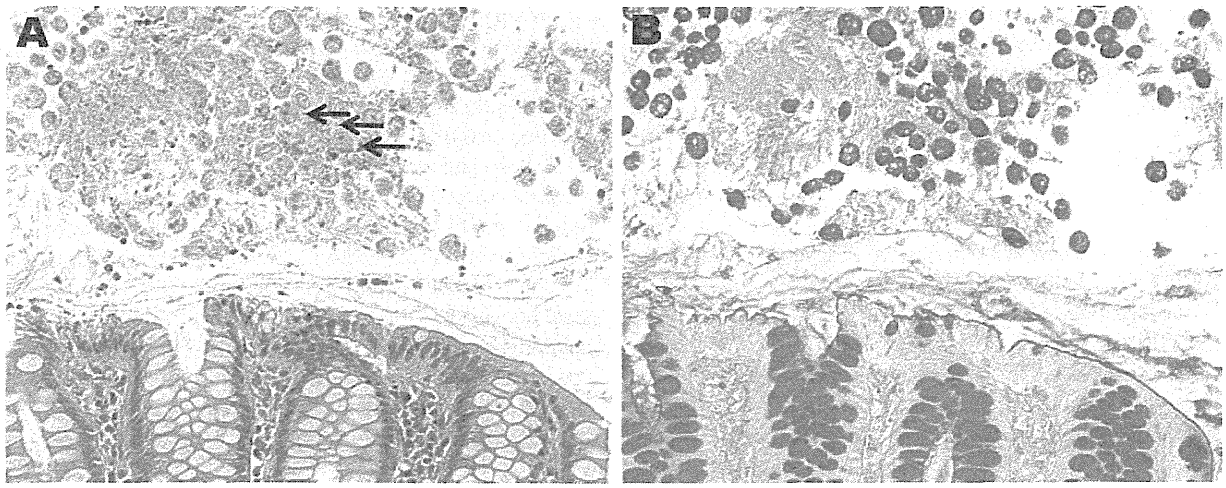

Figure 2. Histologic analysis of amebic colitis, Japan, 2003–2009. A) Trophozoites of *Entamoeba histolytica* ingesting erythrocytes (arrows) (hematoxylin and eosin stain). B) Numerous amebic trophozoites on the mucosal surface (periodic acid–Schiff stain). Original magnification ×200.

analyses were performed by using Stata version 10 software (StataCorp LP, College Station, TX, USA).

Results

Annual Prevalence of Amebic Colitis

Among 10,930 patients, 54 (0.5%) showed development of amebic colitis. Prevalence was 0.16% in 2003 but tended to increase over time ($p<0.01$ by trend test) (Figure 3). Prevalence was 5.6-fold higher in 2009 than in 2003.

Patient Characteristics

HIV-infected patients constituted 248 (2.3%) of 10,930 patients, and they had a median age of 43 years (interquartile range [IQR] 35–55 years) (Table 1). These HIV-infected patients were predominantly male (91.5%, 227/248). Median CD4 cell count was 230 cells/μL (IQR 89.5–401 cells/μL). Routes of HIV infection included homosexual (58.9%, 146/248), heterosexual (12.5%, 31/248), bisexual (10.5%, 26/248), unknown (12.1%, 30/248), untreated blood products (6.0%, 15/248), and drug use (0%).

Patients with a history of HBV infection constituted 184 (1.7%) of 10,390 patients, and they had a median age of 61 years (IQR 47.5–69 years). These patients were also predominantly male (69.0%, 127/184).

Patients with a history of syphilis constituted 266 (2.4%) of 10,390 patients, and they had a median age of 64 years (IQR 48–74 years). These patients were also predominantly male (76.3%, 203/266).

Risk Factors for Amebic Colitis

Risk factors for amebic colitis were age <50 years (OR 11.4, 95% CI 6.1–22.4), male sex (OR 18.5, 95% CI 4.9–156.7), HIV infection (OR 66.2, 95% CI 36.6–120.7), history of HBV infection (OR 9.0, 95% CI 3.4–20.4) and history of syphilis (OR 19.6, 95% CI 10.2–36.2) (Table 1). Multivariate analysis showed that age <50 years (OR 4.73, 95% CI 2.43–9.20, $p<0.001$), male sex (OR 8.39, 95% CI 1.99–35.40, $p<0.01$), HIV infection (OR 15.85, 95% CI 7.93–31.70, $p<0.01$), and history of syphilis (OR 2.90, 95% CI 1.40–5.99, $p<0.01$) were independent risk factors for amebic colitis. This logistic regression model was evaluated by using the Hosmer-Lemeshow test ($p = 0.44$) and receiver operating characteristic area under the curve (0.90).

Comparison of HIV-Positive and HIV-Negative Patients

Annual Prevalence of Amebic Colitis

Numbers of HIV-positive and HIV-negative patients have been increased annually during 2003–2009 in Japan (Figure 4). Among HIV-positive patients, the prevalence in 2009 increased by 2.1-fold over that in 2003 (Figure 4, panel A). Among HIV-negative patients, the prevalence in 2009 increased by 7.1-fold over that in 2003 (Figure 4, panel B).

Risk Factors for Amebic Colitis

Among HIV-positive patients, age <50 years, history of syphilis, and MSM status were risk factors for amebic colitis (Table 2). Immunosuppressed status, such as CD4 cell count <100 cells/μL, was not associated with amebic colitis among HIV-positive patients (Table 2). As CD4 cell counts decreased, the prevalence of amebic decreased (OR 0.3; $p = 0.08$ by trend test).

Among HIV-negative patients, age <50 years, male sex, history of HBV infection, and history of syphilis were risk factors for amebic colitis (Table 2). No interactions were apparent between HIV infection and risk factors, such as age, sex, history of syphilis, and history of HBV infection.

Route of Amebic Infection

Among HIV-positive patients, all 31 patients with amebic infection were male (Table 3). Of these patients, 28 were MSM and 2 were male CSWs. No patients reported contact with CSWs. The route of infection was unknown for 3 patients.

Among HIV-negative patients, 2 patients were female and 21 were male. Both female patients were CSWs. Of the 21 male patients, 8 had had sexual contact with a female CSW and 7 patients were MSM (2 bisexual and 5 homosexual). The route of infection was unknown for 6 patients.

Discussion

Endoscopic examination combined with biopsy sample collection is a valuable method for confirming suspected amebic colitis, which is often misdiagnosed as inflammatory bowel disease or other forms of infectious colitis caused by the similarity of associated gastrointestinal symptoms (e.g.,

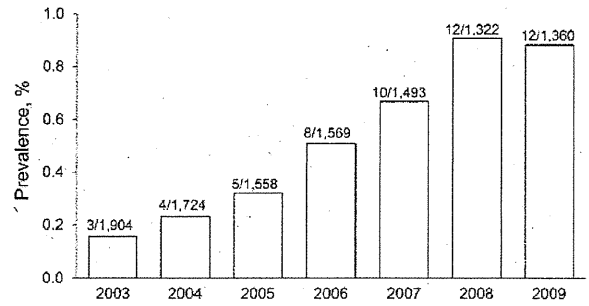

Figure 3. Annual prevalence of amebic colitis, Japan, 2003–2009. Values above bars are no. positive/no. tested.

Table 1. Characteristics and risk factors for 10,930 patients with amebic colitis, Japan, 2003–2009*

Characteristic	All, n = 10,930	Amebic colitis, n = 54	No amebic colitis, n = 10,876	Odds ratio (95% CI)
Median age (IQR)	64 (54–73)	41 (36–52)	65 (54–73)	NA
Age, y				
≥50	8,875 (81.2)	15 (27.7)	8,860 (81.5)	Referent
<50	2,055 (18.8)	39 (72.2)	2,016 (18.5)	11.4 (6.1–22.4)
Sex				
F	4,522 (41.4)	2 (3.7)	4,520 (39.1)	Referent
M	6,408 (58.6)	52 (96.3)	6,356 (58.4)	18.5 (4.9–156.7)
HIV infection				
Negative	10,682 (97.7)	23 (42.5)	10,659 (98.0)	Referent
Positive	248 (2.3)	31 (57.4)	217 (2.0)	66.2 (36.6–120.7)
HBV infection				
Negative	10,746 (98.3)	47 (87.0)	10,699 (84.0)	Referent
Positive	184 (1.7)	7 (13.0)	177 (1.6)	9.0 (3.4–20.4)
Syphilis				
Negative	10,664 (97.6)	37 (68.5)	10,627 (97.7)	Referent
Positive	266 (2.4)	17 (31.5)	249 (2.3)	19.6 (10.2–36.2)

*Values are no. (%) except as indicated. IQR, interquartile range; NA, not applicable; HBV, hepatitis B virus. p values for all comparisons were <0.05, by Mann-Whitney U test.

diarrhea, hematochezia, and abdominal pain) (*14,22,23*). However, only a few studies have included patients who had undergone endoscopy (*17,22,23*). In the present study, we performed a large number of endoscopic examinations. The prevalence of patients with amebic colitis was 0.5% (54/10,930) in this 7-year study. This prevalence was far lower than results from serum prevalence studies, which have shown prevalence in children of 8.4% in Mexico (*24*) and 4.2% in Bangladesh (*25*). However, the annual prevalence of the disease showed a tendency to increase to nearly 1% in recent years, and we assume the prevalence will continue to increase in the future.

In the past, amebic infection in Japan was reportedly caused by overseas travel to countries where epidemics occurred or where amebic infection was found in residents of facilities for the intellectually disabled (*16,26*). However, patients with these characteristics were not observed in this study. Multivariate analysis indicated that risk factors for amebic colitis in this study were male sex, age <50 years, and histories of syphilis and HIV infection.

The reason male sex was a risk factor might be related to specific sexual preference (*8,10–15*) because 52 male patients with amebic colitis often had contact with MSM (n = 35) or female CSWs (n = 8). In this study, MSM constituted 90% of men (OR 4.7 for patients with HIV infection), which is consistent with results of previous reports (*8,10–15*). However, HIV-negative male patients included heterosexual patients, and ≈35% of them had had contact with CSWs. We included CSWs as routes of infection for amebiasis because amebiasis among female CSWs has been reported in Japan (*27*). Therefore, new infection routes other than MSM, which has been considered a risk because of a diversity of sexual activities, should be considered.

Consistent with results of past reports (*8,14,15*), younger age was a risk factor. One possibility is that younger age represents a risk factor because younger persons are more sexually active, although this was not clarified in the present study.

Histories of syphilis or HIV infection have been noted as risk factors in previous case series (*7,15,28*). The present study included many patients with HIV infection or history of syphilis, which supports the hypothesis that these factors increase the risk for amebic colitis.

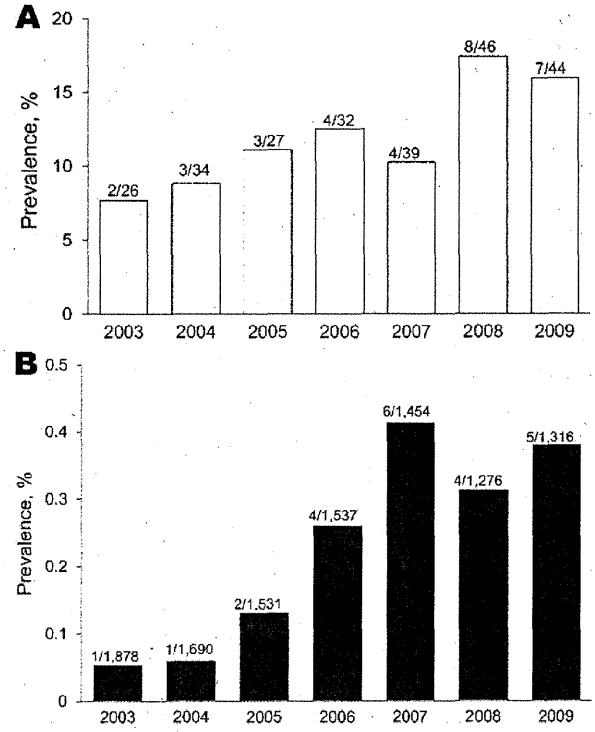

Figure 4. Annual prevalence of amebic colitis in persons with or without HIV infection, Japan, 2003–2009. A) HIV-positive patients. B) HIV-negative patients. Values above bars are no. positive/no. tested.

Table 2. Risk factors for amebic colitis among HIV-positive and -negative patients, Japan, 2003–2009*

Risk factor	HIV-positive patients: Amebic colitis, n = 31	HIV-positive patients: No amebic colitis, n = 217	HIV-positive patients: OR (95% CI)	HIV-positive patients: p value	HIV-negative patients: Amebic colitis, n = 23	HIV-negative patients: No amebic colitis, n = 10,659	HIV-negative patients: OR (95% CI)	HIV-negative patients: p value	p value for interaction
Age, y									
≥50	6	83	Referent		9	8,777	Referent		
<50	25	134	2.6 (1.0–8.0)	0.04	14	1,882	7.3 (2.9–19.0)	<0.01	0.11
Sex									
F	0	21	Referent		2	4499	Referent		
M	31	196	4.6† (0.8–∞)	0.11†	21	6,160	7.7 (1.9–67.5)	<0.01	0.56
HBV infection									
Negative	26	189	Referent		21	10,510	Referent		
Positive	5	28	1.3 (0.4–3.8)	0.62	2	149	6.7 (0.8–27.9)	<0.01	0.07
Syphilis									
Negative	16	163	Referent		21	10,464	Referent		
Positive	15	54	2.8 (1.2–6.5)	<0.01	2	195	5.1 (0.6–21.1)	0.01	0.48
Sexual preference									
Heterosexual	3	73	Referent		ND	ND	ND	ND	ND
MSM	28	144	4.7 (1.4–25.0)	<0.01	ND	ND	ND	ND	ND
CD4 cell count/μL									
>300	14	82	Referent	ND	ND	ND	ND	ND	ND
201–300	5	31	0.9 (0.3–2.8)	ND	ND	ND	ND	ND	ND
100–200	9	39	1.35 (0.5–3.4)	ND	ND	ND	ND	ND	ND
<100	3	65	0.3 (0.07–1.0)	0.15	ND	ND	ND	ND	ND

*OR, odds ratio; HBV, hepatitis B virus; ND, no applicable data; MSM, men who have sex with men.
†Analysis by using exact logistic regression model because number in cell was 0.

Among STDs, HIV infection showed the highest risk ratio, a ≈16-fold increase. HIV infection has been identified as a risk factor for invasive amebiasis in many studies (*10–12,21*), although many details of this risk remain unclear (*19,29*).

We presumed that compromised immune function increased the susceptibility of patients to invasive diseases. However, no relationship was seen between low CD4 cell counts and development of amebic colitis. Under existing conditions, the reason for HIV infection representing a risk factor for amebic colitis is considered the preference for oral–anal sex as a common risk factor for both infectious conditions.

We compared prevalence and risk factors between amebic colitis patients with and without HIV infection. An incidence of 0.1% (4/5,193) has been reported in studies of HIV-negative patients with positive results for occult blood in feces (*17*), and our results were similar. However, annual prevalence increased in 2009 (0.38%, 5/1,316) compared with 2003 (0.05%, 1/1,878), and the rate of increase was higher than that for HIV-positive patients. This result calls for careful attention in hospitals in which patients with HIV infection are not commonly encountered. In terms of risk factors, ORs for age, sex, and history of HBV infection or syphilis in our study did not vary according to HIV infection status.

Table 3. Route of amebic infection for 54 persons, Japan, 2003–2009*

Route	HIV positive, no. (%), n = 31	HIV negative, no. (%), n = 23
Travelers from tropical areas	0	0
Residents of facilities for intellectually disabled	0	0
MSM, male CSW	28, 2 (90.3)	7 (30.4)
Female CSW	0	2 (8.7)
Contact with female CSW	0	8 (34.8)
Unknown	3 (9.7)	6 (26.1)

*MSM, men who have sex with men; CSW, commercial sex worker.

Some limitations need to be considered in this study. First, Japan has not had epidemics of amebiasis, and data in this study were obtained from a metropolitan area. In addition, our hospital treats the largest number of patients with HIV infection in Japan. Second, selection bias was present because participants were patients who had undergone endoscopic examinations, which are highly likely to be performed for healthy patients. In addition, patients suspected before examination of having amebiasis might have been more likely to be actively included in the study. Third, the number of patients with amebic colitis was small; thus, the statistical power of the study might have been low. Fourth, a retrospective design was used for this investigation. With regard to HBV infection or history of syphilis, judgments had to be made for using results of serologic testing in some cases. In addition, determination of sexual preferences and overseas travel had to be based on the self-reports of patients.

In recent years, infectious diseases caused by *E. histolytica* and HIV have been increasing in Japan (*15,16,30*). HIV infection is a particularly serious problem because its incidence is consistently increasing in Japan while decreasing in western countries (*30,31*).

Numbers of patients with both infectious diseases studied are predicted to increase because little is known about measures to prevent infection in association with

a diversity of sexual activities. Amebic infection, in particular, is scarcely recognized as a sexually acquired infection, and improved education is needed to prevent these diseases. In Japan, measures to prevent the spread of HIV and amebic infections are urgently needed.

In conclusion, although this study was conducted at 1 center and involved retrospective analysis of a relatively small number of cases of amebic infection, the results suggest that the number of amebic colitis patients with or without HIV infection is tending to increase in Japan. Younger men with syphilis and HIV infections are at increased risk for amebic colitis. Route of infection differed slightly in that contact with CSWs was more frequent among HIV-negative patients than among HIV-positive patients. Among HIV-positive patients, homosexual intercourse, and not immunosuppressed status, seems to be a risk factor for amebic colitis.

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Dr. Nagata is a gastroenterologist at the NCGM in Tokyo, Japan. His research interests include gastrointestinal infections such as esophageal candidiasis, cytomegalovirus-related disease, mycobacterial infections, intestinal amebiasis, intestinal spirochetosis, chlamydial infection, and HIV-related gastrointestinal disease.

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Parasitology

Diagnostic accuracy of indirect immunofluorescence assay for intestinal invasive amebiasis and impact of HIV infection in a non-endemic country☆,☆☆

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ABSTRACT

To diagnose amebic colitis (AC), serologic tests have the advantage of being inexpensive, noninvasive, and easy to perform, but few studies have investigated their utility, especially in a non-endemic country. A total of 299 symptomatic patients (165 HIV-infected patients) who underwent endoscopy and indirect immunofluorescence (IF) assay were analyzed between 2003 and 2009. The diagnosis of AC was defined as detection of amebic trophozoites from biopsy specimens or intestinal fluid sample via endoscopy. Forty-five patients (29 HIV-infected patients) were diagnosed with AC. The area under the receiver-operating characteristic curve (ROC-AUC) for the IF assay was excellent (0.90), and a cut-off value of 100 provided 89% sensitivity and 87% specificity. ROC-AUC was slightly lower in patients with HIV infection (0.88) than in those without HIV infection (0.94). Among HIV-infected patients, ROC-AUC showed no significant differences between different CD4+ cell counts. The IF assay is useful for diagnosing AC in symptomatic patients with and those without HIV infection.

1. Introduction

Entamoeba histolytica, the causative agent of invasive amebiasis, is responsible for approximately 40,000–110,000 deaths annually around the world (Stanley, 1996, 2003). Although Japan is a non-endemic country, the prevalence of amebiasis has been increasing as that of sexually transmitted diseases (STDs) (Nagata et al., 2012a,b; Ohnishi and Murata, 1997; Ohnishi et al., 2004; Watanabe et al., 2011).

The most frequent manifestations of invasive amebiasis are colitis and liver abscess (Allason-Jones et al., 1986; Petri and Singh, 1999; Stanley, 2003). The trophozoite invades the colonic intestinal epithelium, followed by extra-intestinal spread to the peritoneum, liver, and other sites (Haque et al., 2003; Stanley, 2003). Diagnosis of amebic colitis (AC) in the early phase is thus important. Unfortunately, early diagnosis of AC is often difficult, as the differential diagnoses for the typical gastrointestinal (GI) symptoms (e.g., acute diarrhea, dysentery) are broad and include infectious and noninfectious colonic diseases.

Endoscopy can be a valuable tool in the diagnosis of AC, because visual examination of the colon using endoscopy with biopsy or aspiration of intestinal fluid can help identify mucosal abnormalities and exclude other causes of GI symptoms (Fotedar et al., 2007; Haque et al., 2003; Nagata et al., 2012a,b; Petri and Singh, 1999; Proctor, 1991; Stanley, 2003; Tanyuksel and Petri, 2003). However, endoscopy cannot be recommended for all patients with GI symptoms because of considerations of cost and invasiveness. On the other hand, a number of noninvasive diagnostic tests have been developed for the detection of invasive amebiasis (Fotedar et al., 2007; Haque et al., 2003; Petri and Singh, 1999; Proctor, 1991; Stanley, 2003; Tanyuksel and Petri, 2003). Among these, antiamebic serologic tests have the advantage of being inexpensive, noninvasive, and easy to perform (Fotedar et al., 2007; Proctor, 1991; Tanyuksel and Petri, 2003). Serologic tests are particularly useful in the setting of amebic liver abscess (Fotedar et al., 2007; Proctor, 1991; Tanyuksel and Petri, 2003), but only a limited number of studies have investigated the utility of these tests for AC and very few studies have been undertaken in a non-endemic country (Abd-Alla et al., 1998, 2000; Huston and Petri, 1999).

In addition, there have been suggestions that human immunodeficiency virus (HIV)–infected patients may develop severe invasive

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amebiasis (Mitarai et al., 2001; Seeto and Rockey, 1999). Due to alterations in immune status, there may be differences in serologic test results between HIV-positive and -negative patients, and the effects of HIV infection on the diagnostic accuracy of serologic tests remain unknown.

The goal of this study was to identify the diagnostic accuracy of a serologic test for AC in patients who undergo endoscopy and to assess the differences in test accuracy between HIV-positive and -negative patients.

2. Materials and methods

2.1. Participants

A total of 317 symptomatic patients with suspected AC who underwent colonoscopy at the National Center for Global Health and Medicine (NCGM, Tokyo, Japan) between 2003 and 2009 were reviewed retrospectively. "Suspected cases" were defined as those showing distinctive endoscopic findings such as aphthae, erosions, ulcers, or exudates, as previously reported (Nagata et al., 2012a,b). Antiamebic serologic testing was performed on all patients with identified suspected cases.

Patients who had already received antiamebic treatment prior to endoscopy were excluded from the study. Patients with amebic liver abscess diagnosed by abdominal ultrasonography or computed tomography were likewise excluded. NCGM is a 900-bed hospital and the largest referral center for HIV/AIDS in Japan.

2.2. Ethics statement

The institutional review board at NCGM approved this study. All patients from whom clinical samples were obtained during endoscopy or biopsy provided written informed consent prior to endoscopy. No ethical problems exist with regard to the publication of this manuscript. We used anonymized data from patient medical records.

2.3. Clinical factors

GI symptoms (e.g., diarrhea, bloody stool, loose stool, and abdominal pain) were determined from medical records written by attending physicians who interviewed each patient prior to endoscopy. Laboratory data regarding STDs such as hepatitis B virus infection, syphilitic infection, and HIV infection were recorded before endoscopy.

When a diagnosis of AC was suspected, the doctor asked the patient directly for information related to the route of amebic infection. The doctor clarified the sexual orientation of the patient (i.e., men who have sex with men [MSM] or heterosexual) and whether the patient had travelled to tropical areas or was a resident of a facility for the mentally disabled. For travel-related exposure, history of overseas travel in the past year was elicited. Sexual orientation was confirmed by medical staff or a doctor who questioned each patient face to face. Patients to whom none of the above applied were treated as unknown cases.

For HIV-positive patients, CD4+ cell count within 1 week prior to endoscopy was recorded. We categorized CD4+ cell counts into 4 groups: <100, 100–199, 200–299, and ≥300 cells/μL.

2.4. Diagnosis of AC due to E. histolytica infection

Colonoscopy was performed in patients who presented with GI symptoms within the prior month. When encountering distinctive endoscopic findings of AC (Fig. 1A and B), endoscopic biopsy was performed and intestinal fluid was aspirated from the lesion. "Confirmed cases" of AC were defined as 1) detection of amebic trophozoites from biopsy specimens stained using hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) stains (Fig. 2A and B) or from intestinal fluid samples (Fig. 2C); 2) negative intestinal fluid culture for bacterial species or acid-fast bacillus; and 3) negative histologic features for other colonic diseases (Fotedar et al., 2007; Haque et al., 2003; Petri and Singh, 1999; Proctor, 1991; Stanley, 2003; Tanyuksel and Petri, 2003).

2.5. Anti–E. histolytica antibody test

Data collected within 1 month after the onset of GI symptoms were evaluated. Presence of anti-*E. histolytica* antibody was assessed by indirect immunofluorescence (IF) assay (Ameba-Spot IF; bioMerieux, Marcy l'Etoile, France), as described previously (Mithal et al., 1978). Serum antibody titers <100 were considered negative, while titers of 100, 200, 400, 800, 1600, and 3200 were considered positive.

2.6. Statistical analysis

Patients were classified into 1 of 2 groups based on the presence or absence of AC, and clinical characteristics were compared between groups. A chi-square test was used to compare the frequencies of patient characteristics. A Mann–Whitney *U* test was used to compare age and CD4+ cell counts between groups.

Area under the receiver-operating characteristic curve (ROC-AUC) analysis was used to quantify the accuracy of antiamebic serologic testing. Sensitivity, specificity, and likelihood ratios (LRs) for the diagnosis of AC were also calculated for different cutoff values (serum antibody titers of <100, 100, 200, 400, 800, 1600, and 3200) as previously reported (Mithal et al., 1978).

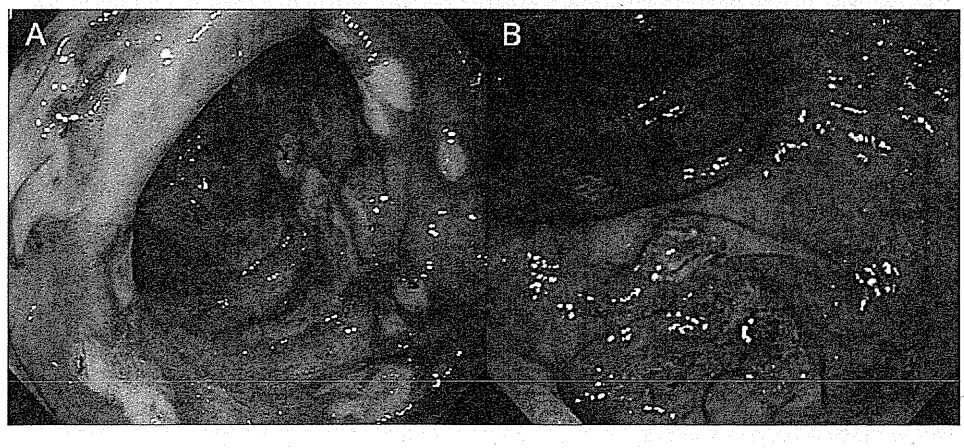

Fig. 1. Distinctive endoscopic findings of amebic colitis. (A) Multiple ulcerous lesions are present in exudates in the sigmoid colon. (B) Multiple erosive lesions with bleeding are seen in the cecum.

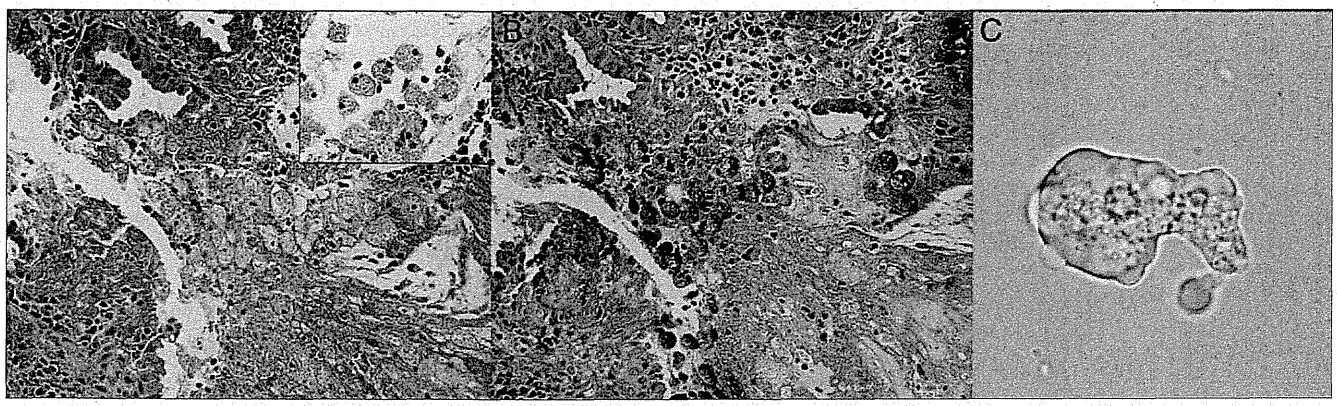

Fig. 2. Detection of *Entamoeba histolytica*. (A) Trophozoites specific for *E. histolytica* could be determined by their size, nuclear morphology, and by the phagocytosis of erythrocytes in hematoxylin and eosin sections. (B) Histologic examination with periodic acid-Schiff stain more clearly reveals numerous amebic trophozoites on the mucosal surface. (C) Light microscopic examination of intestinal fluid aspirated endoscopically sometimes reveals trophozoites of *E. histolytica*.

Diagnostic accuracy using ROC-AUC was also used in subgroup analyses to identify differences in serologic test results in the presence/absence of HIV infection. In HIV-infected patients, differences in ROC-AUC were compared among different CD4 categories.

Values of $P < 0.05$ were considered significant. All statistical analyses were performed using the Stata version 10 software (StataCorp, College Station, TX, USA).

3. Results

3.1. Patient characteristics

Of the 317 patients, 18 were excluded from analysis, including 11 who had already received antiamebic treatment and 7 who showed amebic liver abscess. Thus, a total of 299 patients were selected for analysis. Among these 299 patients, 45 patients (15%) were diagnosed with AC. Patient characteristics are shown in Table 1. Male sex and syphilitic infection were significantly more prevalent in patients with AC than in those without AC ($P = 0.03$ and $P < 0.01$, respectively). MSM orientation was more prevalent in patients with AC than in those without AC ($P < 0.01$). Patients with AC did not include any individuals who had traveled to tropical areas or who were residents of facilities for the mentally disabled.

3.2. Diagnostic accuracy of anti-E. histolytica antibody test

The ROC-AUC of the serologic test was 0.90 (95% confidence interval [CI], 0.85–0.95) (Fig. 3). The accuracy of the diagnostic testing for AC is shown in Table 2. Using a serum antibody titer cut-off of 100 for a positive serologic test yielded 89% sensitivity, 87% specificity, and a LR of 3.95 for the diagnosis of AC. Five patients with AC were serologically negative and 33 patients without AC were serologically positive.

3.2.1. Diagnostic accuracy of the serologic test with or without HIV infection

In subgroup analyses, the ROC-AUC for serologic testing was slightly lower in patients with HIV infection (0.88; 95% CI, 0.80–0.95) than in patients without HIV infection (0.94; 95% CI, 0.87–1.00) (Fig. 4), but this difference did not reach the level of statistical significance ($P = 0.27$). Differences in the diagnostic accuracy of the serologic test for AC in patients with or without HIV infection are shown in Table 3.

3.2.2. Serologic test in different immune statuses among HIV-infected patients

Among HIV-infected patients, no trends in ROC-AUC for the serologic test were seen among the different CD4 categories (Table 4; $P = 0.26$). Serum positive antibody titer was not significantly correlated with CD4 category (Spearman's rho $= -0.12$, $P = 0.52$) in AC patients with HIV infection (Fig. 5).

4. Discussion

This study investigated the accuracy of the Ameba-Spot IF assay in diagnosing endoscopically confirmed AC and also characterized the

Table 1
Clinical characteristics of 299 patients with suspected amebic colitis (AC).

	With AC ($n = 45$)	Without AC ($n = 254$)	*P* value
Median age, years (IQR)	43 (34–57)	41 (36–52)	0.31
Male sex	43 (95.6%)	210 (82.7%)	0.03
Sexual orientation			
Heterosexual	5 (11.1%)	127 (50.0%)	<0.01
MSM	27 (60.0%)	77 (30.3%)	
Unknown	13 (28.9%)	50 (19.7%)	
STD infection			
HBV infection	4 (8.89%)	20 (7.87%)	0.82
Syphilitic infection	15 (33.3%)	39 (15.4%)	<0.01
HIV infection	29 (64.4%)	136 (53.5%)	0.18
Median CD4+[a] cell count (IQR)	300 (150–403)	188.5 (47–358)	0.02
MSM[b]	26 (89.7%)	70 (51.5)	<0.01

IQR = Interquartile range; MSM = men who have sex with men.
[a] CD4+ counts.
[b] MSM were analyzed among HIV-infected patients.

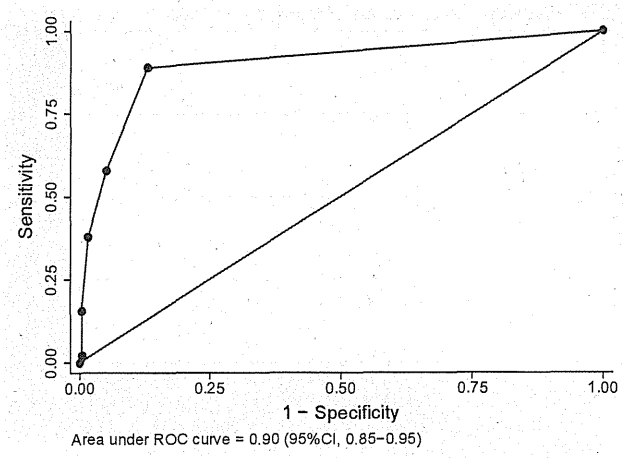

Fig. 3. Diagnostic accuracy of anti-*E. histolytica* antibody test.

Table 2
Diagnostic accuracy of the Ameba-Spot IF assay for amebic colitis (AC).

Serum antibody titer	All patients ($N = 299$)		Sensitivity (95% CI)	Specificity (95% CI)	LR (95% CI)
	With AC ($n = 45$)	Without AC ($n = 254$)			
<100	5	221	NA	NA	0.13 (0.06–0.29)
100	14	20	88.9% (79.0–94.5)	87.0% (77.4–93.6)	3.95 (2.16–7.24)
200	9	9	57.8% (42.1–74.4)	94.9% (82.7–99.4)	5.64 (2.37–13.4)
400	10	3	37.8% (18.1–61.6)	98.4% (83.9–100)[a]	18.8 (5.39–65.7)
800	6	0	15.6% (0.32–52.7)	99.6% (63.1–100)[a]	72.1[b] (4.13–1257)
1600	1	1	2.22% (0–84.2)[a]	99.6% (15.8–100)[a]	5.64 (0.36–88.6)

CI = Confidence interval; LR = likelihood ratio; NA = not applicable.
IF assay results are presented as titer values of <100, 100, 200, 400, 800, 1600, and 3200. Sensitivity and specificity are presented as the cut-off value for each titer.
[a] One-sided, 97.5% confidence interval.
[b] Likelihood ratios were estimated using the substitution formula. 0.5 was added to all cell frequencies before calculation.

Table 3
Differences in diagnostic accuracy of the Ameba-Spot IF assay for amebic colitis (AC) in patients with or without HIV infection.

Serum antibody titer	HIV-infected patients ($n = 165$)		Sensitivity (95% CI)	Specificity (95% CI)	LR (95% CI)
	With AC ($n = 29$)	Without AC ($n = 136$)			
<100	4	113	NA	NA	0.07 (0.01–0.46)
100	6	13	86.2% (72.2–93.9)	83.1% (69.8–92.5)	2.16 (0.90–5.22)
200	5	6	65.5% (45.7–82.1)	92.7% (77.2–99.2)	3.91 (1.28–11.9)
400	9	3	48.3% (26.0–74.0)	97.1% (72.7–99.9)	14.1 (4.06–48.8)
800	4	0	17.2% (0.42–64.1)	99.3% (54.1–100)[a]	41.1[b] (2.27–743)
1600	1	1	3.45% (0–84.2)[a]	99.3% (15.8–100)[a]	4.69 (0.30–72.8)
Serum antibody titer	Non-HIV-infected patients ($n = 134$)		Sensitivity (95% CI)	Specificity (95% CI)	LR (95% CI)
	With AC ($n = 16$)	Without AC ($n = 118$)			
<100	1	108	NA	NA	0.07 (0.01–0.46)
100	8	7	93.8% (74.0–99.0)	91.5% (74.0–99.0)	8.43 (3.53–20.1)
200	4	3	43.8% (12.2–73.8)	97.5% (69.2–100)[a]	9.83 (2.42–40.0)
400	1	0	18.8% (0.84–90.6)	100% (0.84–90.6)	21.0[b] (0.89–495)
800	2	0	12.5% (0–84.2)[a]	100% (1.26–98.7)	35.0[b] (1.75–698)

IF assay results are presented as titer values of <100, 100, 200, 400, 800, 1600, and 3200. Sensitivity and specificity are presented as the cut-off value for each titer.
[a] One-sided, 97.5% confidence interval.
[b] Likelihood ratios were estimated using the substitution formula. 0.5 was added to all cell frequencies before calculation.

differences in the accuracy of this test for patients with and without HIV infection. In previous studies, serologic testing for AC patients has been associated with variable sensitivity and specificity (Abd-Alla et al., 1998, 2000; Fotedar et al., 2007; Huston and Petri, 1999; Petri and Singh, 1999; Proctor, 1991; Tanyuksel and Petri, 2003). For example, Huston and Petri (1999) reported that the sensitivity of the indirect hemagglutination assay (IHA) was >90% in AC patients, while Abd-Alla et al. (1998) reported that the sensitivity of IgG enzyme-linked immunosorbent assay (ELISA) ranged from 31% to 93.1% and that the specificity of the IgG ELISA was 92.9% for AC. Another article from Abd-Alla et al. (2000) reported that the sensitivity of serum IgG ELISA ranged from 56.3% to 100% and that the specificity of serum IgG ELISA was 76.6% for AC. According to their data, sensitivity and specificity decreased when symptoms were present for less than a week when compared with symptoms lasting for more than a week. Although the present study did not include duration of symptoms as a variable, all patients underwent serologic testing and endoscopy within 1 month after symptom onset. We found that a serum antibody titer ≥100 was associated with the highest sensitivity (88.9%) and with good specificity (87.0%). To the best of our knowledge, this represents the first study of the Ameba-Spot IF assay that describes its accuracy in the diagnosis of AC.

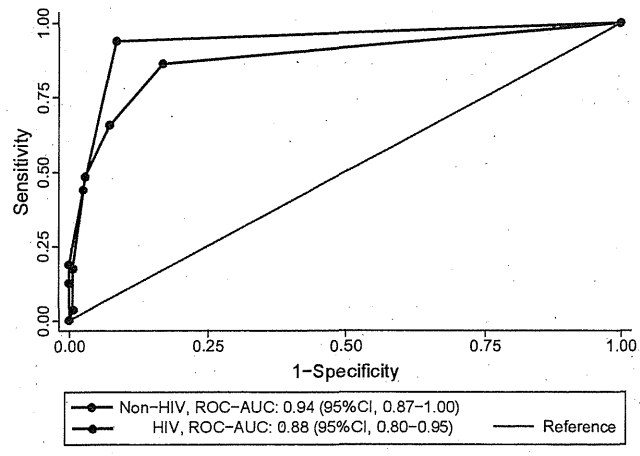

Fig. 4. Diagnostic accuracy of antibody test in patients with or without HIV infection.

Some caution should be taken in interpreting the application of serologic testing in clinical practice. First, antibodies can persist for years after infection (Fotedar et al., 2007; Petri and Singh, 1999; Proctor, 1991). Serologic testing alone thus cannot distinguish current from past infections and may lead to false-positive results. In this study, 33 patients showed false-positive results. We speculate that patients with STDs have a high risk and high rate of recurrence for amebiasis (Hung et al., 2005; Watanabe et al., 2011), leading to an increased possibility of false-positive results. We therefore investigated differences in the prevalence of STDs between serologically positive and negative non-AC patients ($n = 254$). Serologically positive patients tended to have STDs more frequently (16.6%) than serologically negative patients (8.7%), but this difference did not reach the level of statistical significance ($P = 0.06$).

Second, appropriate antibodies may not be generated for 2–4 weeks after infection, which can lead to false-negative results early in the course of the illness (Fotedar et al., 2007; Huston and Petri, 1999,

Table 4
Differences in ROC-AUC of the Ameba-Spot IF assay for amebic colitis among different CD4 categories in HIV-infected patients ($n = 165$).

CD4 category	Number	ROC-AUC	95% CI
<100 cells/μL	52	0.79	0.39–1.00
100–199 cells/μL	26	0.96	0.90–1.00
200–299 cells/μL	29	0.82	0.67–0.97
≥300 cells/μL	58	0.89	0.77–1.00

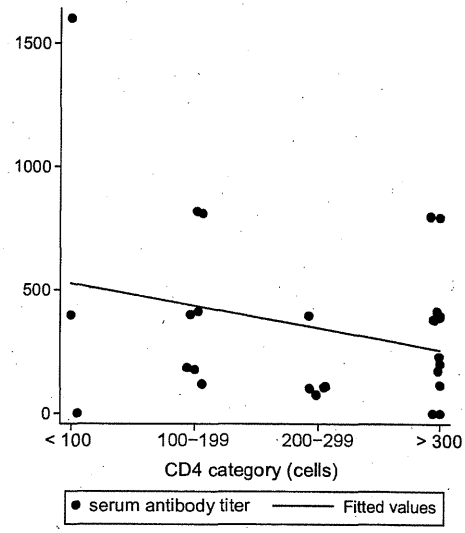

Fig. 5. Serum antibody titer and CD4+ cell counts in amebic colitis patients with HIV infection (n = 29).

1991; Petri and Singh, 1999; Proctor, 1991;). Indeed, 5 patients showed false-negative serologic results in this study. In each of these cases, a review of the endoscopic images revealed only mild cecal lesions. Mild AC of less than 2 weeks' duration may thus result in a false-negative result from the serologic test.

Third, the accuracy of serologic testing is significantly influenced by the seroprevalence rates in endemic areas (Fotedar et al., 2007; Petri and Singh, 1999; Proctor, 1991; Tanyuksel and Petri, 2003), because there is no cross-reaction with non–*E. histolytica* parasites and the specificity of the test should be high (Fotedar et al., 2007). Seroprevalence rates should thus be kept in mind when interpreting serologic data. Although Japan is a non-endemic country, the prevalence of amebiasis has been increasing as that of STDs (Nagata et al., 2012a,b; Ohnishi and Murata, 1997; Ohnishi et al., 2004; Watanabe et al., 2011). Furthermore, recent studies have shown that patients who are male, are <50 years old, have a history of syphilitic infection, and have HIV infection were independent risk factors for invasive amebiasis (Nagata et al., 2012a,b). Our results should thus be particularly applicable to patients at high risk for STDs in Japan.

Fourth, patients with amebic liver abscess diagnosed by abdominal ultrasonography or computed tomography were excluded in this study to identify the accuracy of intestinal amebiasis. However, 37 patients had not undergone ultrasonography and computed tomography because they presented without fever, but all showed negative results on serologic testing. We thus believe that the accuracy of serologic assays had little influence, since patients with amebic liver abscess would be more likely to present with detectable levels of antibodies (Fotedar et al., 2007; Proctor, 1991; Tanyuksel and Petri, 2003).

Although a higher prevalence of *E. histolytica* infection is seen among patients with HIV infection (Hung et al., 2005; Ohnishi and Murata, 1997; Ohnishi et al., 2004; Watanabe et al., 2011), the diagnostic accuracy of serologic testing for AC when comparing patients with and without HIV infection has remained unclear. In this study, the accuracy of serologic testing for AC was slightly lower in patients with HIV infection than in patients without HIV infection. HIV infection–related immunosuppression results in impaired antibody formation, which could adversely affect the sensitivity of serologic testing for AC. Indeed, this finding could be attributable to the differences in immune status between HIV-positive and -negative patients. However, our results from HIV-infected patients indicated no correlation between the ROC-AUC of serologic tests and CD4+ cell counts. Moreover, we found that serum antibody titer was not significantly correlated with CD4+ cell counts in AC patients with HIV infection. A significant difference might not have been confirmed due to the small number of AC patients with HIV infection. Further studies with a greater number of patients are needed to clarify this issue. Moreover, HIV-infected MSM had a high prevalence of repeat amebic infection (Watanabe et al., 2011), which could have contributed to the lower specificity of serologic testing for AC.

In conclusion, the Ameba-Spot IF assay is useful for diagnosing AC in symptomatic patients with and those without HIV infection. However, the result of slightly lower accuracy of the IF assay in patients with HIV infection than in those without HIV infection should be interpreted with care. No significant differences in test results were seen between different immune statuses as determined by CD4+ cell counts. These results are particularly applicable to patients at high risk for STDs in a non-endemic country.

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MAJOR ARTICLE

Entamoeba moshkovskii Is Associated With Diarrhea in Infants and Causes Diarrhea and Colitis in Mice

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Background. *Entamoeba moshkovskii* is prevalent in developing countries and morphologically indistinguishable from pathogenic *Entamoeba histolytica* and nonpathogenic *Entamoeba dispar*. It is not known if *E. moshkovskii* is pathogenic.

Methods. Mice were intracecally challenged with the trophozoites of each *Entamoeba* spp. to test the ability to cause diarrhea, and infants in Bangladesh were prospectively observed to see if newly acquired *E. moshkovskii* infection was associated with diarrhea.

Results. *E. moshkovskii* and *E. histolytica* caused diarrhea and weight loss in susceptible mice. *E. dispar* infected none of the mouse strains tested. In Mirpur, Dhaka, Bangladesh, *E. moshkovskii*, *E. histolytica*, and *E. dispar* were identified in 42 (2.95%), 66 (4.63%), and 5 (0.35%), respectively, of 1426 diarrheal episodes in 385 children followed prospectively from birth to one year of age. Diarrhea occurred temporally with acquisition of a new *E. moshkovskii* infection: in the 2 months preceding *E. moshkvskii*-associated diarrhea, 86% (36 of 42) of monthly surveillance stool samples were negative for *E. moshkovskii*.

Conclusions. *E. moshkovskii* was found to be pathogenic in mice. In children, the acquisition of *E. moshkovskii* infection was associated with diarrhea. These data are consistent with *E. moshkovskii* causing disease, indicating that it is important to reexamine its pathogenicity.

Entamoeba histolytica causes extensive mortality and morbidity worldwide through diarrheal disease and abscess formation in parenchymal tissues such as liver, lung, and brain. In contrast, other amoebae that infect humans include *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba coli*, *Entamoeba hartmanni*, and *Endolimax nana*, which have been considered nonpathogenic commensals of the human gut [1–3]. *Dientamoeba fragilis* and *Entamoeba polecki* have been associated with diarrhea and *Entamoeba gingivalis* with periodontal disease [4, 5].

E. moshkovskii is genetically related to *E. histolytica* and *E. dispar* and is microscopically indistinguishable from them in its cyst and trophozoite forms [6]. This species of *Entamoeba* was first identified in sewage in Moscow by Tshalaria in 1941 [7] and was initially thought to be a free-living common protozoan species in anoxic sediments and in environments such as brackish coastal pools. The first human isolate was obtained from a resident of Laredo, Texas, who suffered from diarrhea, weight loss, and epigastric pain in 1961 [8]. This finding would seem to suggest and/or support that *E. moshkovskii* can be pathogenic. At first, this isolate was named *E. histolytica* Laredo strain and shared biological features with *E. moshkovskii*.

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Both the Laredo strain and *E. moshkovskii* grow at room temperature and were resistant to osmotic shock and to drugs used in the chemotherapy of amoebiasis such as emetine [9]. Subsequent molecular studies revealed that *E. histolytica* Laredo is identical with *E. moshkovskii* [10].

E. moshkovskii is a common *Entamoeba* infection in humans in some settings. It is composed of anywhere from as little as 1% to as high as 50% of the *E. histolytica/E. dispar/E. moshkovskii* complex parasites detected in fecal samples in limited studies from Australia, Bangladesh, India, Iran, Tanzania, and Turkey [6, 11–16]. These studies for the most part tested stool samples submitted to clinical microbiology laboratories from patients with gastrointestinal symptoms, suggesting that *E. moshkovskii* could cause disease. However, in HIV-1–infected individuals in northern Tanzania, *E. moshkovskii* was not associated with enteric symptoms nor immune status [17]. Thus, the ability of *E. moshkovskii* to cause disease in humans remains unclear.

Here we tested the ability of *E. moshkovskii* to cause colitis and diarrhea in a murine model system, in which intracaecal inoculation with *E. histolytica* trophozoites into CBA/J, C3H/HeN, and C3H/HeJ mice leads to amebic colitis [18–20]. In addition, we tested in a longitudinal study of children in Bangladesh not only if *E. moshkovskii* was present in stool samples from infants with diarrhea, but whether the *E. moshkovskii* infection was newly acquired at the time of the diarrheal illness.

MATERIALS AND METHODS

Mice

Male CBA/J, C57BL6/J, BALB/c, C3H/HeN, and C3H/HeJ mice were purchased from the Jackson Laboratory. Animals were maintained under specific pathogen-free conditions at Animal Research Center for Tropical Infectious Diseases, Nagasaki University, and were challenged when they were 5–8 weeks old.

Cultivation of *Entamoeba* spp.

Trophozoites of the *E. moshkovskii* Laredo strain were a gift from Dr Seiki Kobayashi, Keio University, School of Medicine (originally from the late Professor Louis S. Diamond, National Institutes of Health, Bethesda, Maryland). Trophozoites of *E. histolytica,* originally laboratory strain HM1:IMSS (American Type Culture Collection, Manassas, Virginia), were from Professor Eric Houpt, University of Virginia, which were sequentially passaged in vivo through the mouse cecum [18]. Cecal contents were cultured at 25° and 37°C, respectively, in BIS-33 medium supplemented with heat-inactivated 10% adult bovine serum, 25 U/mL penicillin, and 25 mg/mL streptomycin [21]. Trophozoites of *E. dispar* AS16IR were also provided by Dr Seiki Kobayashi and cultured in YIMDHA-S media at 37°C. Trophozoites under log phase of growth were used in the experiments.

Intracecal Inoculation of *Entamoeba* spp.

Trophozoites were harvested from culture tubes of *E. histolytica* HM1:IMSS, *E. moshkovskii* Laredo and *E. dispar* AS16IR strains by incubating the tubes on ice for 5–10 minutes. Then, the trophozites were collected, and the number of trophozoites was determined. We anesthetized mice with domitor (medetomidine hydrochloride: 0.1 mg/kg) and dormicum (midazolam: 0.1 mg/kg), shaved their abdomens to incise the skin and exteriorized each cecum from the peritoneum, and injected 150 μL of 1×10^6 each trophozoites into the proximal, middle, and apical sites of cecum. Then the cecum was blotted and the peritoneum and the skin were sutured. Mice were kept on warming blankets at 37°C throughout. Survival rates were ≥85% in all strains. The study was approved by the animal ethical review board of Nagasaki University.

PCR Amplification for Diagnosis of *Entamoeba* spp. Infection in Mice

For isolation of *Entamoeba* DNA from mouse stools, QIAamp DNA Stool kits (QIAGEN, Valencia, California) were used according to manufacturer's instructions. The primer sequences used for polymerase chain reaction (PCR) were described elsewhere [22].

Pathology of Murine Amoebic Colitis

At the indicated days after intracecal challenge, mice were killed, the ceca fixed in phosphate buffered 10% formalin, and then cut into 4–6 equal cross-sections and embedded in paraffin, and 4 μm slides were stained with H&E.

Child Study Area and Population

The study was conducted in Mirpur, an urban slum in Dhaka. Infants were enrolled in the first week after birth and followed until one year of age, beginning in January 2008. Field research assistants (FRAs) visited each study house every other day and collected information related to child morbidity, especially for diarrheal illness, through a structured questionnaire. If the FRA found any child with an acute illness, then she referred the child to the study clinic for further management by the medical officer. Parents or guardians were also encouraged to visit the study clinic for medical assistance if the study child became sick. FRAs collected nondiarrheal monthly stool specimens as well as diarrheal stool specimens from the home or in the study field clinic. All stool specimens were transported from the field to the clinic using a cold box. In the field clinic an aliquot of the diarrheal stool specimens was placed into Carry-Blair medium. All specimens were transported from the field clinic to the ICDDR,B Parasitology laboratory within 3 hours of collection, with a cold chain maintained. Diarrhea was defined as having ≥3 unformed or abnormal stools (as per the mother's perception) in a 24-hour period. A diarrheal episode was defined as being separated from another episode by at least 3 diarrhea-free days.

The study was approved by the Institutional Review Board of the University of Virginia, and the Ethical Review Committee of the International Centre for Diarrhoeal Disease Research, Bangladesh. Informed written consent was obtained from the parents or guardians for the participation of their child in the study.

Detection of Enteropathogens

Stool samples were cultured for enteric pathogens including *Vibrio cholerae* O1/O139, *Salmonella* spp., *Shigella* spp., and *Campylobacter jejuni*. Enzyme-linked immunosorbent assay (ELISA) methods were used to detect LT and ST producing enterotoxigenic *E. coli* (ETEC) [23]. *Entamoeba histolytica, Cryptosporidium,* and *Giardia* were identified by real-time PCR as described elsewhere [24]. Rotavirus, astrovirus, and adenovirus were detected by ELISA using commercial kits (ProSpectT Rotavirus Catalog R240396, ProSpectT Astrovirus Catalog R240196, and ProSpectT Adenovirus Catalog R240096, respectively). Multiplex (RT-)PCR and probe-based detection with Luminex beads for conceivable diarrhea-causative microbes was performed as described elsewhere in the literature [25–27].

The DNA was extracted using a slightly modified QIAamp DNA Stool Mini Kit protocol (Qiagen Inc, Valencia, California) [24]. The RNA was extracted using the QuickGene RNA tissue kit SII [25, 26]. For the (RT-)PCR-Luminex assay, either the forward or the reverse primer per target was labeled with biotin-TEG at 5′ ends. After (RT-)PCR was performed with the conditions described elsewhere, samples were analyzed on the BioPlex-200 system using bead on which coupling and hybridization were performed according to published protocols [28].

Amplification of Arg^{TCT} Gene Fragment and Sequencing

The *E. moshkovskii*–specific primer pair, EmR-1 and EmR-2, was used to specifically amplify the *E. moshkovskii* Arg^{TCT} gene fragment [13]. Amplification was performed using the high-fidelity Sahara DNA polymerase (Bio-Line, US). Sequencing was performed on an Applied Biosystems 377 Prism DNA Sequencer, using the BigDye terminator chemistry and EmR-1 or EmR-2 primer.

Statistical Analysis

The χ^2 test and Mann–Whitney *U* test were used where they were applicable.

RESULTS

E. moshkovskii Established the Infection in Mice

We previously showed that C3H/HeN, C3H/HeJ, and CBA/J mice allowed the establishment of *E. histolytica* infection, whereas many strains of mice including C57BL/6 and BALB/c mice did not, indicating that susceptibility to *E. histolytica* infection depended on the genetic background of the host [18–20]. Trophozoites of either *E. histolytica, E. moshkovskii,* or *E. dispar* were intracecally injected into congenic strains of mice. *E. histolytica* successfully infected the ceca of C3H/HeN, C3H/HeJ, and CBA/J mice. *E. moshkovskii* infected the ceca of CBA/J mice in approximately 68% (51 of 75) of mice at 4 days after challenge, as determined by both culture and PCR of intracecal contents. Likewise, C3H/HeN and C3H/HeJ mice were infected with *E. moshkovskii* in 60% and 40% of cases at 4 days, respectively, whereas infection rates of C57BL/6 and BALB/c mice were 5.6% and 0.0% at 4 days, respectively (Table 1). Nonpathogenic *E. dispar* did not infect any mouse strain tested. These data demonstrated that in contrast to nonpathogenic *E. dispar* that did not infect, *E. moshkovskii* had a similar host genetic susceptibility to infection in the murine model as did pathogenic *E. histolytica*.

Table 1. Susceptibility of Congenic Strains of Mice to *Entamoeba histolytica, Entamoeba moshkovskii,* or *Entamoeba dispar* Infection

	E. histolytica (%)	*E. moshkovskii* (%)	*E. dispar* (%)
BALB/c	1/15 (6)	0/10 (0)	0/10 (0)
C57BL/6	2/20 (10)	1/18 (6)	0/15 (0)
C3H/HeJ	8/15 (53)	4/10 (40)	0/10 (0)[a]
C3H/HeN	7/15 (47)	6/10 (60)	0/10 (0)[a]
CBA/J	61/90 (68)	51/75 (68)	0/20 (0)[a]

[a] $P < .05$ compared to *E. histolytica* or *E. moshkovskii* (χ^2 test).

E. moshkovskii Induced Intestinal Symptoms in Mice

Intestinal symptoms and body weight were monitored after challenging CBA/J mice with *E. moshkovskii*. A total of 71% (51/72) of CBA/J mice inoculated with *E. moshkovskii* were infected by 3 days after challenge. Diarrhea was observed in 39% (20/51) and dysentery in 6% (3/51) (Figure 1*A–C*). In successfully infected mice, amoebae were observed in the lumen of the ceca (Figure 1*D*). Mice with bloody diarrhea exhibited a thickened and contracted ceca (Figure 1*E*). Histopathological examination of ceca from these mice revealed epithelial ulceration, hemorrhagic changes, and tissue destruction (Figure 1*F*). Furthermore, obvious weight loss was observed during the course of *E. moshkovskii* infection in CBA/J mice, which was more severe than that observed during pathogenic *E. histolytica* infection, both of which were significant compared to control sham-operated mice (Figure 1*H*). Together these data indicated that *E. moshkovskii* was virulent in mice.

E. moshkovskii Was Expelled Within 14 Days After Challenge, Whereas *E. histolytica* Chronically Infected in the Ceca of Mice

The time course of each *Entamoeba* spp. infection in susceptible strains of mice was observed. As was reported [20], *E. histolytica* established chronic infection in not only CBA/J

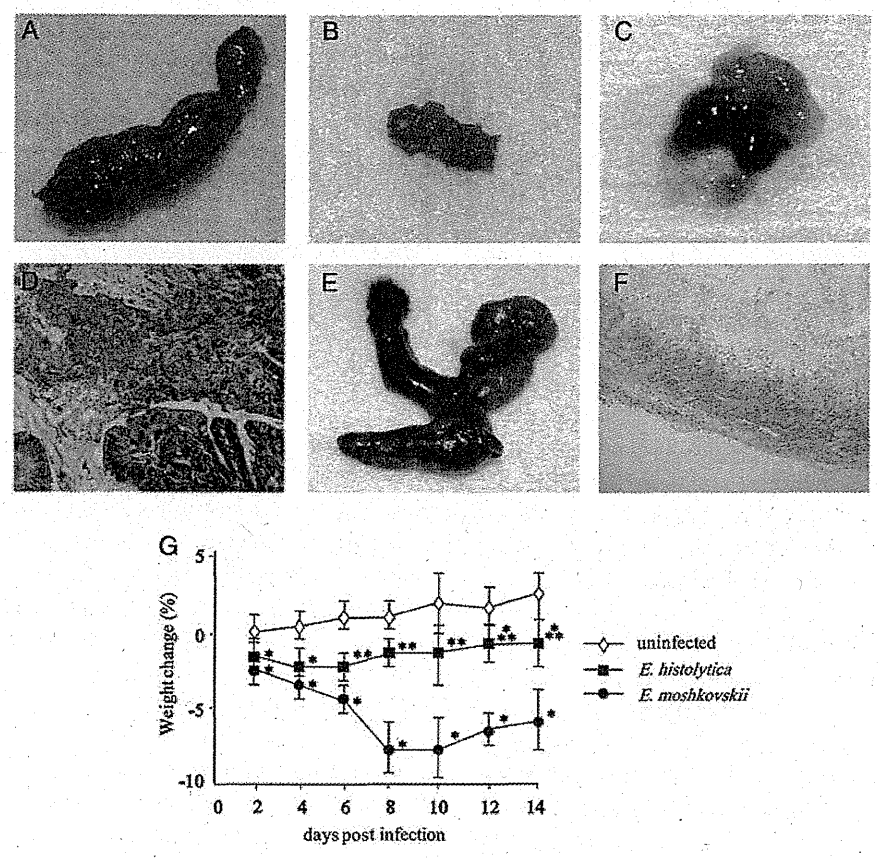

Figure 1. *Entamoeba moshkovskii* induced intestinal symptoms and weight loss in CBA/J mice. CBA/J mice were intracecally inoculated with 1×10^6 trophozoites of *E. moshkovskii*. After infection, diarrhea, colitis, and weight loss were monitored. Normal (*A*), loose (*B*), and bloody feces (*C*) were observed as was indicated in the results. Amoebae were observed in the lumen of the ceca in successfully infected mice (*D*). Macroscopic and histopathological observations of ceca in mice exhibited bloody diarrhea were shown in panels *E* and *F*. Changes in body weight were monitored in successfully infected 15 mice per group (*G*), in which CBA/J mice were intracecally inoculated with 1×10^6 trophozoites of *Entamoeba histolytica* (*solid squares*), *E. moshkovskii* (*solid circles*), or medium alone (*open diamonds*). The study was repeated 3 times with similar results. $^*P < 1.0 \times 10^{-6}$, $^{**}P < 1.0 \times 10^{-5}$ and $^{***}P < 1.0 \times 10^{-4}$ compared with sham-operated mice (Mann–Whitney *U* test).

but also C3H/HeJ and C3H/HeN mice, whereas neither C57BL/6 nor BALB/c allowed establishment of *E. histolytica* infection (Figure 2). In contrast, *E. moshkovskii* did not cause chronic infection, being expelled by approximately 2 weeks after challenge in CBA/J mice (Figure 2). A similar time to clearance was seen in C3H/HeN and C3H/HeJ mice.

In Infants in Bangladesh, *E. moshkovskii* Was Detected in Diarrheal Samples With Similar Frequency to *E. histolytica*

The association between diarrheal episodes and infection with each *Entamoeba* spp. was tested in children in Mirpur, Dhaka, Bangladesh. These studies were part of a prospective cohort study on diarrheal diseases [29]. Newborn children were enrolled in the Mirpur community of Dhaka, Bangladesh, and prospectively followed for diarrheal illness by every other day home visits. A total of 1426 diarrheal episodes were recorded during the first 12 months of life in 385 children. PCR analyses of the diarrheal samples revealed that 66 episodes were positive for *E. histolytica* (4.63%), 42 were positive for *E. moshkovskii* (2.95%), and 5 episodes were positive for *E. dispar* (0.35%). As such, in diarrheal samples, the detection rates of either *E. histolytica* or *E. moshkovskii* were 13.2 and 8.4 times higher than that of nonpathogenic *E. dispar*. Two episodes were found to be mixed infections with *E. histolytica* and *E. moshkovskii*, but no other mixed infections of *Entamoeba* spp. were found.

E. moshkovskii Infection Was Newly Acquired in Children With Diarrhea

In order to attempt to discern if the *E. moshkovskii* detected in the diarrheal stool sample could be the cause of diarrhea, we tested if it was newly acquired at the time of diarrhea. The preceding 2 months of surveillance stool samples collected when the child did not have diarrhea were tested for the

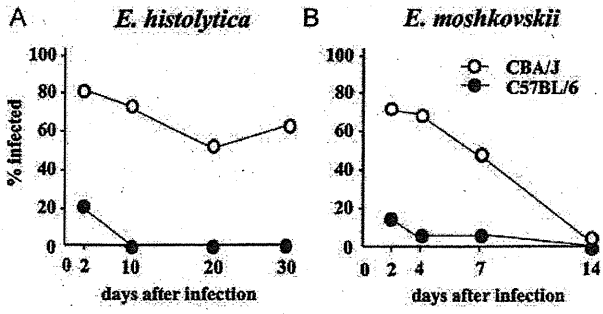

Figure 2. *Entamoeba moshkovskii* was expelled within 2 weeks in CBA/J Mice. CBA/J (open circles) and C57BL/6 (solid circles) mice were intracecally inoculated with 1×10^5 trophozoites of *Entamoeba histolytica* (*A*) or *Entamoeba moshkovskii* (*B*). Time course of each *Entamoeba* spp. infection was then monitored by detection of the parasites in stool by culture and polymerase chain reaction (PCR).

presence of *E. moshkovskii*. This study design therefore temporally controlled for *E. moshkovskii* infection in the 42 infants with diarrhea attributed to this parasite. In the 1 and 2 months preceding *E. moshkvskii*-associated diarrhea, 93% (39/42) and 86% (36/42) of monthly surveillance stool samples, respectively, were negative for *E. moshkovskii* (Table 2). This supported the hypothesis that temporal acquisition of a new *E. moshkovskii* infection led to diarrheal episodes in some proportion of these children.

E. moshkovskii-Associated Diarrhea Was of Similar Severity to Other Causes of Diarrhea

The diarrheal severity score was comparable among episodes associated with *E. histolytica, E. moshkovskii,* and other causes: 4.89 ± 0.22, 4.71 ± 0.24, and 4.84 ± 0.05, respectively. The duration of diarrhea was also comparable among these episodes positive for *E. histolytica, E. moshkovskii,* and others: 4.44 ± 0.44, 4.74 ± 0.49, and 4.84 ± 0.10 days, respectively (Table 3). The mean age of the onset of diarrheal episodes associated with *E. histolytica, E. moshkovskii,* and others was found to be 7.72 ± 0.75, 9.12 ± 0.73, and 9.09 ± 0.18 months, respectively, without any significant differences. Thus, the diarrhea related to *E. moshkovskii* was indistinguishable from diarrhea related to *E. histolytica* in severity, duration, and age of onset (Table 3).

Table 2. Prevalence of *Entamoeba moshkovskii* Asymptomatic Infection Preceding *E. moshkovskii*-Associated Diarrhea in 42 Children

Category	Preceding 1-Month Surveillance Stool	Preceding 2-Month Surveillance Stool
E. moshkovskii (+)	3	6
E. moshkovskii (–)	39	36
Total	42	42

Table 3. Severity and Duration of Diarrhea Associated With *Entamoeba histolytica* or *Entamoeba moshkovskii*

Pathogens	Severity Score (mean ± SE)	Duration (days) (mean ± SE)	Age of Onset in Months (mean ± SE)
E. histolytica	4.89 ± 0.22	4.44 ± 0.44	7.72 ± 0.75
E. moshkovskii	4.71 ± 0.24[a]	4.74 ± 0.49[a]	9.12 ± 0.73[a]
Others	4.84 ± 0.05	4.84 ± 0.10	9.09 ± 0.18

[a] No significant difference in diarrheal severity score or duration for episodes associated with *E. histolytica, E. moshkovskii,* or other enteropathogens infection.

Additional Enteropathogens Were Identified in Stool Samples From *E. moshkovskii* Infected Children

As there are many microbes that can potentially induce diarrhea, the presence of other diarrheagenic microbes was tested in the 42 diarrheal samples that were associated with *E. moshkovskii*. The 42 samples were examined for other conceivable diarrhea-causative microbes infection using standard bacterial culture techniques, fecal antigen detection, and multiplex PCR combined with probe-based detection with Luminex beads (Table 4) [25, 26]. In the 42 diarrheal stool samples with *E. moshkovskii,* 12 samples (28.6%) contained >4 other pathogens, 13 (31.0%) had 3 pathogens, 14 (33.3%) had 2 pathogens, 1 (2.3%) had 1 pathogen, and 2 samples were positive solely for *E. moshkovskii* (Table 4). The application of these state-of-the-art diagnostic techniques in this cohort has on average identified a minimum of 2 different enteropathogens in every diarrheal stool sample (E. Houpt and M. Taniuchi, personal communication, 2011). It was therefore not surprising that the diarrheal episodes associated with *E. moshkovskii* were commonly coinfected.

E. moshkovskii Isolates Were Genetically Diverse in the Infants

In order to investigate the genetic diversity in *E. moshkovskii* strains detected in the infected children's stools, we used a tRNA-gene linked locus (R-R), which previously showed PCR size differences among *E. moshkovskii* strains from Bangladesh [12, 30]. Twenty-six *E. moshkovskii*-positive stool DNAs (6 from asymptomatic children and 20 from diarrheal children) were amplified using the *E. moshkovskii* specific nested PCR primers described elsewhere[12]. However, PCR did not reveal any obvious product size differences among these samples (data not shown). Because same size PCR products do not necessarily mean identical DNA sequences, we sequenced PCR products directly without cloning them into any vectors (in order to minimize the chances of any sequence selection) to detect sequence variation. Sequencing did reveal that the

Table 4. Other Enteropathogens Detected in *Entamoeba moshkovskii* (+) Diarrheal Stool Samples

Name of Organism	No. of Samples
Encephalitozoon intestinalis	1
Cyclospora cayetanensis	1
Cystoisospora belli	1
Enterocytozoon bieneusi	6
Adenovirus	1
Astrovirus	4
Sapovirus	2
Norovirus G1	0
Norovirus G2	4
Rotavirus	1
E. histolytica	2
Giardia intestinalis	10
Cryptosporidium spp.	1
Vibrio cholera/parahaemolyticus	3
EAEC	15
ETEC	3
EPEC	5
EHEC	0
EIEC/Shigella spp.	23
Salmonella (pan)	2
Aeromonas (pathogenic)	14
Yersinia (pan)	0
Campylobacter jejuni/coli	23

Table 5. Single-Nucleotide Polymorphisms (SNPs) in the *E. moshkovskii* Strains from Bangladesh at Locus R-R

ID	Clinical Status	No. of SNPs	Position and SNP Type
7040-CDS05	Diarrhea	6	T204A, C205T, T206C, C208del, T209del, T210del
7063-CDS02	Diarrhea	1	T71C
7161-CDS05	Diarrhea	1	T141G
7146-CDS02	Diarrhea	1	A235T
8119-CDS02	Diarrhea	2	T83C, T137C
8113-CDS04	Diarrhea	3	T81C, C120T, G221A
7086-CMS15	Asymptomatic	2	T203W, T204Y
8056-CMS15	Asymptomatic	1	A135R

All positions are based on the consensus sequence in the alignment. W = A/T; Y = C/T; R = A/G.

E. moshkovskii strains detected in this study were polymorphic in locus R-R; although unlike the *E. histolytica* and *E. dispar* sequences[31], no short tandem repeats could be detected in *E. moshkovskii*. Single-nucleotide polymorphisms (SNPs) were detected in 2 of the 6 asymptomatic children-derived sequences and in 6 of the 20 diarrheal children-derived sequences (Supplementary Figure 1). These SNPs could be used to divide them into 9 different genotypes—18 strains with identical locus R-R sequences and the remaining 8 strains containing ≥1 distinct SNPs (Supplementary Figure 1 and Table 5). Because we used a high-fidelity DNA polymerase (Bio-Line, US) during PCR amplification, it was unlikely that these SNPs were erroneously introduced by the DNA polymerase. The sequence alignment at locus R-R revealed that the *E. moshkovskii* strains of this study were comparatively more diverse than the reference *E. moshkovskii* Laredo strain, but closer to the only Bangladeshi strain (ID:MS15-3646) sequenced previously (labeled as Em-Laredo and Em-BANGLA, respectively, in Supplementary Figure 1). The SNPs detected in this study were distributed randomly across the locus R-R sequences, and as a result, these SNPs could not be used to differentiate asymptomatic and diarrheal strains of *E. moshkovskii*. However, we noticed from the sequence traces that the 2 asymptomatic strains (IDs:8056-CMS15 and 7086-CMS15) showed allelic variation in all 3 SNPs (T203W, T204Y, and A135R), whereas none of the 6 diarrheal strains showed any allelic variations in their respective SNPs (Table 5 and Supplementary Figure 2). The significance of this remains unknown at present.

DISCUSSION

This work draws into question the paradigm that *E. moshkovskii* is avirulent. In the murine model of intestinal amebiasis, *E. moshkovskii* caused diarrhea, weight loss, and colitis. In this way, *E. moshkovskii* shared with *E. histolytica*, but not the nonpathogen *E. dispar*, the ability to cause disease. In children in Bangladesh, the new acquisition of *E. moshkovskii* infection was associated with diarrhea.

E. moshkovskii infected the ceca of C3H/HeN, C3H/HeJ, and CBA/J mice, but not C57BL/6 or BALB/c mice, which was consistent with the host range of pathogenic *E. histolytica*. In contrast, the nonpathogenic parasite *E. dispar* was unable to infect the intestine of any strains of mice tested. The finding that *E. moshkovskii* shared with *E. histolytica* the ability to infect mice indicates that they share virulence mechanisms, which are not present in *E. dispar*.

Mouse strain-dependent resistance to *E. histolytica* infection was mediated by nonhematopoietic cells [19]. Relatively few loci on C57BL/6 chromosomes 1 and 2 correlated with resistance to intestinal amebiasis [32]. In humans, one important means of innate resistance of intestinal epithelial cells to amebiasis is leptin, which acts via STAT3 signaling to protect intestinal epithelial cells from parasite killing [33, 34]. In this context, it will be interesting to examine whether this observation is also true in *E. moshkovskii* infection, because of the similar host range as *E. histolytica*. If the mechanism of resistance to *E. histolytica* and *E. moshkovskii* observed in many

inbred strains of mice is shared with humans, identification of regional candidate genes in mice has implications for further understanding the human variability to amebic infection.

E. moshkovskii induced intestinal symptoms including diarrhea and bloody stool, typical symptoms of amebiasis, indicating that *E. moshkovskii* was pathogenically similar to *E. histolytica* at least in mice. Weight loss was also observed during the course of infection, which was more severe in mice infected with *E. moshkovskii* than with *E. histolytica*. The observation that *E. moshkovskii* induced severe intestinal symptoms accompanied by weight loss reemphasizes that it is potentially pathogenic.

However, it is unclear what kinds of differences among *Entamoeba* spp. result in the different outcomes of infection in the murine model. *Entamoeba histolytica* possesses molecules such as pathogen-associated molecular patterns (PAMPs) on its surface that stimulate proinflammatory cytokines production from antigen-presenting cells [35]. We are investigating whether parasite PAMPs, host MyD88 signaling, and the pattern of proinflammatory cytokines produced in response qualitatively differ between *Entamoeba* species, may provide clues to the different severity between CBA/J mice infected with *E. histolytica*, *E. moshkovskii*, and *E. dispar*.

Entamoeba moshkovskii isolates infecting children were genetically heterogeneous, as evidenced by PCR typing of tRNA locus R-R. It will be important in future studies of the potential pathogenicity of *E. moshkovskii* to take into account this heterogeneity; as for the case of *E. histolytica*, not every genotype is equally capable of causing disease [36].

The study subjects reported here differ from those of the previous study examining *E. moshkovskii* infection in preschool children in Dhaka, Bangladesh [13], which was not focused solely on diarrheal stool samples, but also included monthly stool samples from asymptomatic children. In addition, the current study reports on a novel birth cohort longitudinally followed from birth to 1 year of age. Therefore, it is important to discuss the association between diarrhea in infants and *E. moshkovskii* infection in the context of the cohort.

In conclusion, we found that *E. moshkovskii* caused diarrhea, colitis, and weight loss in mice and that in Bangladeshi children acquisition of a new *E. moshkovskii* infection occurred temporally with diarrhea. These data are consistent with *E. moshkovskii* causing diarrhea and indicate that it is important to reexamine its pathogenicity.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. Dr Petri receives royalties from a licensing agreement with TechLab, Inc., for amebiasis diagnostics. These royalties are donated in their entirety to the American Society of Tropical Medicine and Hygiene without benefit to Dr Petri. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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