Paragonimus kellicotti (lung fluke endemic in the United States)
Paragonimus spp.
Metorchis conjunctus (North American liver fluke)

Trematodes—Blood

The schistosomes are acquired by penetration of the skin by the cercarial forms that are released from freshwater snails. Although they are not endemic within the United States, occasionally patients are seen who may have these infections.

Current Name

Schistosoma mansoni
Schistosoma haematobium
Schistosoma japonicum
Schistosoma intercalatum
Schistosoma mekongi

ARTHROPODS

See Tables 208-1 and 208-2.

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The full reference list for this chapter is available at expertconsult.com.

SUBSECTION 1 *PROTOZOA*

CHAPTER 209

AMEBIASIS

Shinjiro Hamano • William A. Petri, Jr.

Diarrheal diseases continue to be major causes of morbidity and mortality in children in developing countries. In Bangladesh, 10 percent of children in the first year of life have amebic diarrhea and 1 in 30 children dies of diarrhea or dysentery by age 5 years.[78] Amebiasis is an infection caused by the protozoan parasite *Entamoeba histolytica*. Infection occurs via ingestion of the parasite's cyst from fecally contaminated food, water, or hands. Approximately 50 million illnesses and 100,000 deaths occur annually from amebiasis, rendering it the third leading cause of death by parasitic disease in humans.[78] Although amebiasis is present worldwide, it occurs most commonly in underdeveloped areas, especially Asia, sub-Saharan Africa, and Central and South America. In the United States and other developed countries, cases of amebiasis are most likely to occur in immigrants from and travelers to endemic regions, but it can affect populations of the developed world, as shown by the epidemic that occurred

in Tbilisi, Republic of Georgia, caused by contaminated municipal water.[13] Currently, there is no vaccine to prevent the childhood morbidity and mortality resulting from infection with *E. histolytica.*

ETIOLOGY

E. histolytica is the cause of amebiasis and was named for the pathologic evidence of "lysis" of tissues. Additional *Entamoeba* spp. that infect humans and that are identical in appearance microscopically to *E. histolytica* include *E. dispar,* which is nonpathogenic, *E. moshkovskii,* which may cause diarrhea,[95] and the recently identified enteric parasite *E. bangladeshi.*[91] The first demonstration of the organism in human tissues was made by Lambl in 1859 in the postmortem examination of the colon of a child who died as a result of having excessive diarrhea.[16,74] No connection of the organism with the disease was made until 1875, when Losch, in St. Petersburg, Russia, found the organism at autopsy in the colon of a woodcutter. Losch induced diarrhea and ulcerations in a dog given feces from the patient.[61] He did not think, however, that a connection existed between the organism and the disease. The first patient described in the United States was a physician treated by Osler for an amebic liver abscess in 1890.[74] Councilman and Lafleur described the organism and the disease in 1891.[20,45] Further investigation of the disease was delayed until a better understanding of the life cycle of *E. histolytica* could be obtained.[27] In recent years, the application of modern molecular biology techniques to the study of *E. histolytica* and *E. dispar* has resulted in an explosion of information about the mechanisms of virulence, pathogenicity, and immune responses to these organisms.[88,90]

E. histolytica is the pathogenic species, having the capacity to invade tissue and cause symptomatic disease, whereas *E. dispar* (and *E. histolytica*) is associated with the asymptomatic carrier state.[6,88] More recently, a study revealed that all genotypes of *E. histolytica* are not equally capable of causing disease.[6] Morphologically distinct members of the genus *Entamoeba,* such as *Entamoeba coli* and *Entamoeba hartmanni,* also are nonpathogenic. *Entamoeba moshkovskii, Dientamoeba fragilis,* and *Entamoeba polecki* have been associated with diarrhea, and *Entamoeba gingivalis* has been associated with periodontal disease.

Members of the genus *Entamoeba,* which are protozoan organisms belonging to the subphylum Sarcodina and close to *Dictyostelium discoideum* on one of the lowest branches of the eukaryotic tree, have trophozoite and cyst forms.[39] The cysts of *E. histolytica, E. dispar; E. moshkovskii,* and *E. bangladeshi* are almost spherical, being surrounded by a cell wall composed of chitin. The cysts may have one to four nuclei, although quadrinucleate cysts are most typical. This feature allows differentiation from *Escherichia coli,* which usually has 6 to 8 nuclei in the cysts and may have 32 nuclei.[75] Cysts of *E. histolytica* are 5 to 20 μm in diameter (average, 12 μm) and have a greenish tint in the unstained condition.[62] Young cysts contain chromatoid bodies, which are composed of ribosome particles in crystalline arrays.[12] The cysts of *E. hartmanni* appear identical to those of *E. histolytica* except for being a smaller size (4 to 10 μm). *E. histolytica* cysts can survive for days in the dried state at 30° C or for months at 0° C to 4° C. They can be killed by temperatures greater than 50° C retained for 5 minutes.[45] They are completely resistant to the concentrations of chlorine used in water supplies but may be killed with hyperchlorination or with iodine solutions.[62,75] They are filtered from water supplies that pass through a sand filtration phase. They resist acids well.

When these quadrinucleate cysts are ingested, they resist the acid pH of the stomach and ultimately excyst in the alkaline environment of the bowel. The process of excystation results in the release of four trophozoites that divide by binary fission to produce eight trophozoites. The usual trophozoites have a diameter of 25 μm (range, 10 to 60 μm).[27,88] They have a single nucleus that is 3 to 5 μm in diameter and contains fine peripheral chromatin with a slightly eccentric karyosome. They have a granular endoplasm that typically contains vacuoles in which bacteria and debris can be seen. Some glycogen is present and can be stained with periodic acid–Schiff stain.

Although amebae were thought to lack organelles, such as mitochondria, endoplasmic reticulum, and Golgi apparatus, evidence to the contrary is coming to light. The existence of nuclear-encoded mitochondrial genes and a remnant mitochondrial organelle was reported more recently.[63,102] The presence of ingested erythrocytes is a characteristic feature of *E. histolytica* but not *E. dispar.*[88] Movement is accomplished by extension of clear pseudopodia. Replication is by binary fission. These protozoa live in the colon of humans and other mammals. Trophozoites die quickly outside the body and are quite sensitive to acid—they generally are not considered to be infective.[32] When cooled (as when feces are expelled and gradually cooled from body temperature) or stimulated by as-yet-undefined luminal conditions, the trophozoites form cysts that can remain viable for weeks to months on excretion.[88]

Trophozoites of *E. coli* are 15 to 50 μm in diameter; have much more sluggish motility than the trophozoites of *E. histolytica;* and have blunt pseudopodia, rather than the sharp, finger-like pseudopodia of *E. histolytica.* Trophozoites of *E. hartmanni* are 4 to 14 μm in diameter and have much less glycogen than the trophozoites of *E. histolytica.*[27]

EPIDEMIOLOGY

Amebiasis is distributed throughout the world. The number of people infected with either *E. histolytica* or *E. dispar* per year is estimated to be 500 million. Although most individuals remain asymptomatic, perpetuating the natural cycle of the organism through fecal excretion of infective cysts, approximately 50 million people experience the severe morbidity associated with invasive disease, with an estimated 100,000 dying annually.[78,99] In the United States, 50 percent of amebiasis is observed in Hispanics, Asians, and Pacific Islanders. Travelers from developing countries, malnourished individuals,[72] men, and residents of institutions for the mentally retarded are considered to be at higher risk for amebiasis (Table 209-1).

TABLE 209-1 Risk Factors for Amebiasis in the United States

Hispanic/Asian/Pacific Islanders—50% of U.S. cases reported to CDC
Travelers to endemic regions of the world—0.3% incidence in one study
Institutions for mentally retarded
Men who have sex with men
Men—90% amebic liver abscesses in men, but rare in children

CDC, Centers for Disease Control and Prevention.

During the 1990s, enough evidence had accumulated to support the formal separation of two morphologically identical species of ameba: the nonpathogenic *E. dispar* from the potentially pathogenic *E. histolytica*.* Morbidity and mortality data in absolute numbers that existed before this time pertaining to cases of invasive disease were not greatly affected by this reclassification because all invasive disease was known to be caused by *E. histolytica*.[99] Because most prevalence and incidence data previously collected pertained to asymptomatic individuals, however, and it was clear that most asymptomatic individuals with cysts detected in their stool were infected with nonpathogenic *E. dispar*, the true prevalence and incidence of *E. histolytica* became a matter of speculation.[99]

Estimates of *E. histolytica* infections have been based primarily on examinations of stool for cysts and parasites, but these tests are insensitive and cannot differentiate *E. histolytica* from morphologically identical species that are nonpathogenic, such as *E. dispar* and *Entamoeba moshkovskii*.[5,22] Specific and sensitive means to detect *E. histolytica* in stool are now available and include antigen detection and polymerase chain reaction (PCR) analysis.[36,44,58]

A prospective study of preschool children in a slum of Dhaka, Bangladesh, showed *E. histolytica*–associated diarrhea in 9 percent and *E. histolytica*–associated dysentery in 3 percent of the children annually.[42] Not all individuals are equally susceptible to amebiasis, with a leptin receptor polymorphism and certain HLA-DR and HLA-DQ alleles associated with resistance to infection and disease.[23,24] The annual incidence of amebic liver abscess was reported to be 21 cases per 100,000 inhabitants in Hue City, Vietnam.[14] Carefully conducted serologic studies in Mexico, where amebiasis is endemic, showed antibody to *E. histolytica* in 8.4 percent of the population.[17] In the urban slum of Fortaleza, Brazil, 25 percent of all individuals tested carried antibody to *E. histolytica;* the prevalence of anti-amebic antibodies in children 6 to 14 years old was 40 percent.[15]

PATHOGENESIS AND PATHOLOGY

The cysts are transported through the digestive tract to the intestine, where they release their mobile, disease-producing form, the trophozoite. *E. histolytica* trophozoites can live in the large intestine and form new cysts without causing disease. They also can invade the lining of the colon, killing host cells and causing diarrhea, amebic colitis, acute dysentery, or chronic diarrhea. The trophozoites also can be carried through the blood to other organs, most commonly the liver and occasionally the brain, where they form potentially life-threatening abscesses (Fig. 209-1). Important virulence factors include the trophozoite cell surface galactose and *N*-acetyl-D-galactosamine (Gal/GalNAc)–specific lectin that mediates adherence to colonic mucins and host cells,[77,89] cysteine proteinases that likely promote invasion by degrading extracellular matrix and serum components, and amebapore pore-forming proteins involved in killing of bacteria and host cells.[59,92]

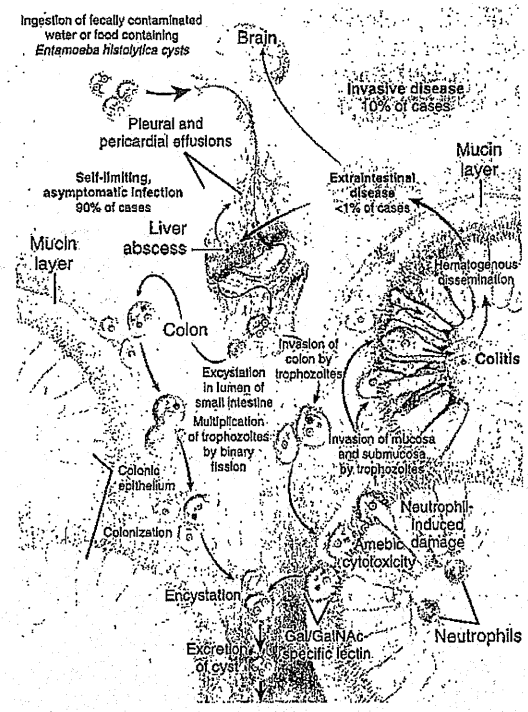

FIGURE 209-1 ■ Life cycle of *Entamoeba histolytica.* Infection normally is initiated by the ingestion of fecally contaminated water or food containing *E. histolytica* cysts. The infective cyst form of the parasite survives passage through the stomach and small intestine. Excystation occurs in the bowel lumen, where motile and potentially invasive trophozoites are formed. In most infections, the trophozoites aggregate in the intestinal mucin layer and form new cysts, resulting in a self-limited and asymptomatic infection. In some cases, adherence to and lysis of the colonic epithelium, mediated by the galactose and *N*-acetyl-D-galactosamine (Gal/GalNAc)–specific lectin, initiates invasion of the colon by trophozoites. Neutrophils responding to the invasion contribute to cellular damage at the site of invasion. When the intestinal epithelium is invaded, extraintestinal spread to the peritoneum, liver, and other sites may follow. Factors controlling invasion, as opposed to encystation, most likely include parasite "quorum sensing" signaled by the Gal/GalNAc-specific lectin, interactions of amebae with the bacterial flora of the intestine, and innate and acquired immune responses of the host. (From Haque R, Huston CD, Hughes E, et al. Amebiasis. *N Engl J Med* 2003;**348**:1565-73.)

*References 1, 14, 30, 31, 38, 99.

The interface of the Gal/GalNAc lectin with the host mucins lining the intestine is the defining moment of the infection.[19] If the parasite lectin attaches to the host mucin glycoproteins that line the intestinal lumen, a noninvasive gut infection ensues. The life cycle continues as the trophozoites reproduce by clonal expansion in the mucin layer. Subsequently, the Gal/GalNAc lectin, along with mucin glycoproteins or other gut bacteria, initiates the developmental pathway leading to encystment.[26,99]

Colitis is caused when the trophozoite penetrates the intestinal mucous layer, which otherwise acts as a barrier to invasion by inhibiting amebic adherence to the underlying epithelium and by slowing trophozoite motility.[19] Invasion is mediated by the killing of epithelial cells, neutrophils, and lymphocytes by trophozoites, which occurs only after the parasite lectin engages host GalNAc on O-linked cell surface oligosaccharides.[78] The interaction of the lectin with glycoconjugates is stereospecific and multivalent.[107] The identity of the high-affinity intestinal epithelial cell receptor is unknown. Secretion of amebapore, a 5-kDa pore-forming protein, by the ameba may contribute to killing.[57] Activation of human caspase 3, a distal effector molecule in the apoptotic pathway, occurs rapidly after amebic contact, and caspases are required for cell killing in vitro and for the formation of amebic liver abscesses in vivo.[47,106]

Interaction of the parasite with the intestinal epithelium causes an inflammatory response marked by the activation of nuclear factor κB and the secretion of cytokines.[25,93] The development of this epithelial response may depend on trophozoite virulence factors, such as cysteine proteinase, and leads to intestinal abnormalities through neutrophil-mediated damage. Neutrophils also can be protective, and activation of neutrophils or macrophages by tumor necrosis factor-α or interferon-γ kills amebae in vitro and limits the size of amebic liver abscesses.[7,21] In contrast to the intense inflammatory response typical of early invasive amebiasis, inflammation surrounding well-established colonic ulcers and liver abscesses is minimal, given the degree of tissue damage.[16]

The initial lesions of clinical amebiasis often are small interglandular ulcers with a diameter of approximately 1 mm. They extend only to the muscularis mucosa.[16,66] The margins may be hyperemic, and slight edema of the surrounding mucosa is present. *E. histolytica* organisms seen in these ulcers stain well with periodic acid–Schiff stain.[79] Bleeding and friability are not prominent at this stage, although proctoscopic examination may find mucus coming from these ulcers, with an abundant number of amebae present.

The next stage of intestinal disease is the production of deeper ulcers. These "buttonhole" ulcers may be 1 cm in diameter and may extend into the submucosa.[16,79] The ulcer often extends laterally under normal-appearing mucosa, forming a characteristic flask shape. Occasional perforation through the serosa leads to peritonitis or pneumoperitoneum.[96] Extensive necrosis may be present, but usually only very little inflammation occurs. The edema is more intense, but the mucosa between ulcers is normal, in contrast to the marked inflammatory response seen in bacterial enteritis. When ulceration is more extensive, the edema surrounding the ulcers becomes confluent and the mucosa appears gelatinous. In young children, this condition can progress to a fulminant necrotizing colitis associated with transmural necrosis. The pathologic events associated with this phenomenon are not understood. Rarely, an inflammatory response is present, resulting in granulation of the tissue with a fibrous outer wall.[75] It is given the name *ameboma*. Occasionally, an ameboma fills a significant portion of the lumen, which causes stricture or obstruction. Other complications of intestinal amebiasis result from direct extension of the ulcers. This extension may result in cutaneous involvement of the perianal area or lesions of the penis, vulva, vagina, or cervix.[2,75] Cutaneous and ophthalmologic amebiasis also is caused by fecal contamination of the face.[69]

Amebae disseminate to the liver in 50 percent of patients with fulminant amebiasis.[2,3] Dissemination to other organs directly from the intestine probably does not occur, but dissemination from the liver to lung, heart, brain, spleen, scapula, larynx, stomach, and aorta has been described.[16] Amebic abscess of the liver occurs more often in men than in women by a ratio of 16:1 but occurs equally often in prepubertal children of both sexes.[3,16] Abscesses occur more commonly in adults but occur in children as young as 4 months of age.[73] These abscesses vary from microscopic lesions to massive necrosis of 90 percent of the liver. Fever, right upper quadrant pain, and the presence of serum antibodies to amebae point to hepatic amebic abscess.[87] Examination of the fluid from such an abscess frequently reveals a reddish, "anchovy paste" fluid that rarely may appear white or green. The fluid is acidic, with a pH ranging from 5.2 to 6.7.[85] Amebae are found in the walls of the abscess and only rarely in the fluid of the abscess. Many patients with amebic liver abscess also have anaerobic bacteria in the abscess fluid.[86] The walls are composed of a thin connective tissue capsule. The right lobe of the liver is involved with amebic liver abscess about six times as often as the left lobe. Abscesses in the right lobe can perforate and cause disease below the diaphragm or in the thoracic cavity. Abscesses in the left lobe can lead to pericardial effusions, which are less common than pleural effusions.[34,49]

Pleural effusions can remain loculated or lead to cutaneous fistulas or to bronchopleural fistulas. Drainage from these fistulas is acidic, in contrast to the neutral secretions in the normal lung. Seeding of the cardiac valves and of the brain has been described.[16] Cerebral abscesses have the same microscopic findings as do liver abscesses, with a thin capsule of connective tissue surrounding a fluid with little or no associated inflammatory response.

IMMUNITY

Protection from amebiasis, including acquired immunity to infection and invasion by *E. histolytica*, is associated with a mucosal IgA antibody response against the carbohydrate recognition domain of the parasite Gal/GalNAc lectin.[33,37,39,55] Cell-mediated immunity in protection

from invasive amebiasis, but not infection per se, also has been shown. There is substantial evidence from an in vitro animal model and most recently from human studies of an important role for interferon-γ and IL-17 in protection from amebic colitis, acting in part by activating of macrophages to kill the parasite.[33,43,46] Invasive amebiasis rarely occurs in individuals with human immunodeficiency virus infection/acquired immunodeficiency syndrome, even in areas where amebiasis is common, suggesting an important role also exists for natural immunity or innate immune responses, or both, in protection from infection.[7,36]

CLINICAL MANIFESTATIONS

Intestinal Amebiasis

Asymptomatic Intraluminal Amebiasis

The most common type of amebic infestation is an asymptomatic cyst-passing carrier state. All *E. dispar* infections and 90 percent of *E. histolytica* infections are asymptomatic, manifesting as only *Entamoeba* cysts in the feces.[30,81]

Entamoeba histolytica*–Associated Diarrhea*

Diarrhea is the most common manifestation of amebic disease, present in 9 percent of children in the Mirpur cohort each year, compared with only 3 percent of children having amebic colitis each year.[42] *E. histolytica*–associated diarrhea is defined as three or more unformed stools in a 24-hour period accompanied by a new episode of *E. histolytica* infection. This definition was validated previously in the cohort by (1) showing that diarrhea was approximately five times more common in the setting of a new infection (age-adjusted odds ratio for the association of new *E. histolytica* infection with diarrhea of 4.7; 95% confidence interval 2.9 to 7.6), and (2) showing by a complete bacteriologic, virologic, and parasitic workup that only 32 percent of patients with *E. histolytica*–associated diarrhea were coinfected with another pathogen compared with identification of an enteropathogen in 59 percent of all cases of diarrhea.[39]

Acute Amebic Colitis

Amebic dysentery was defined as a diarrheal stool sample containing occult or gross blood that was positive for *E. histolytica* antigen. Seventy percent of patients have a gradual onset of symptoms over 3 or 4 weeks after infestation, with increasingly severe diarrhea as the primary complaint, accompanied by general abdominal tenderness. Occasionally, the onset may be acute or may be delayed for several months after infestation. This onset differs from bacterial causes of dysentery, in which patients usually have only symptoms of 1 to 2 days' duration. The diarrhea is usually associated with pain in children. Pain may be of such severity that an acute abdomen is suspected.[50,79] The stools contain blood and mucus in virtually all cases.[2,79,80] Fever is present in only a few patients with amebic colitis. Abdominal distention and dehydration occur in less than 10 percent of patients. In young children, intussusception, perforation, peritonitis, or necrotizing colitis may develop rapidly.[10,50,96] Amebic colitis has been shown to be associated with cognitive disability as a long-term sequelae.[101]

Ameboma

Unusual manifestations of amebic colitis include toxic megacolon (0.5% of cases, usually requires surgical intervention), ameboma (granulation tissue in colonic lumen mimicking colonic cancer in appearance), and a chronic nondysenteric form of infection that can manifest as years of waxing and waning diarrhea, abdominal pain, and weight loss (easily misdiagnosed as inflammatory bowel disease).

Extraintestinal Amebiasis

Amebic Liver Abscess

The typical patient with an amebic liver abscess in the United States is an immigrant, usually a Hispanic/Asian/Pacific Islander; is male; is 20 to 40 years old; and has fever, right upper quadrant pain, leukocytosis, abnormal serum aminotransferase and alkaline phosphatase levels, and a defect on hepatic imaging study. Roughly 90 percent of patients with liver abscesses are men. The abscess usually is single and is in the right lobe of the liver 80 percent of the time.[51] Most frequently, patients have liver abscess without concurrent colitis. Amebae are seen infrequently in the stool at the time of diagnosis of liver abscess.[3] Liver abscess can manifest acutely as fever and right upper abdominal tenderness and pain or subacutely as prominent weight loss and less frequently fever and abdominal pain. The peripheral white blood cell count is elevated, as is the alkaline phosphatase level, in many patients.

Early evaluation of the hepatobiliary system by ultrasonography or computed tomography (CT) is essential to show the abscess in the liver. The differential diagnosis of the lesion in the liver includes pyogenic abscess, hepatoma, and echinococcal cyst. Aspiration of the abscess occasionally is required to diagnose amebiasis (although amebae are visualized in the pus in only a few cases; if the abscess is pyogenic, the responsible bacteria are seen or cultured). Antibodies to *E. histolytica* are present in the serum of 70 to 90 percent of patients on acute presentation with amebic liver abscess and are useful diagnostically, especially in combination with antigen detection or PCR tests.[41] Unusual extraintestinal manifestations of amebiasis include direct extension of the liver abscess to pleura or pericardium and brain abscess. In a patient who has right upper quadrant pain, ultrasonography, CT, or magnetic resonance imaging (MRI) should be performed to examine the liver and gallbladder.

If a space-filling defect in the liver is observed, the differential diagnosis includes (1) amebiasis (most common in men with a history of travel or residence in a developing country); (2) pyogenic or bacterial abscess (suspected in women, patients with cholecystitis, elderly individuals, individuals with diabetes, and patients with jaundice); (3) echinococcal abscess (an incidental finding

because echinococcal abscess should not cause pain or fever); and (4) cancer. Most patients with amebic liver abscess have detectable circulating antigen in serum and serum anti-amebic antibodies.[36]

In children, abdominal pain is reported infrequently with amebic liver abscess.[35,70] More commonly, high fever, abdominal distention, irritability, and tachypnea are noted. Some children are admitted to the hospital with a fever of unknown origin. Hepatomegaly occurs frequently, but elicitation of hepatic tenderness is not well documented. In one report, four of five children younger than age 5 years died with amebic liver abscesses because the diagnosis was not suspected.[56] Death usually results from rupture of the liver abscess into the peritoneum, thorax, or pericardium but may follow extensive hepatic damage and liver failure.[3,84]

Metastatic Amebiasis

Extra-abdominal amebiasis presumably follows direct extension from liver abscesses, rather than direct dissemination from the intestine.[3,16] Thoracic amebiasis is the most common type of extra-abdominal amebiasis and occurs in less than 10 percent of patients with amebic liver abscess.[16,49] Symptoms depend on the type of involvement. Empyema, bronchohepatic fistulas, or extension of a pleuropulmonary abscess into the pericardium may occur.

Pericardial amebiasis is the next most common form of extraintestinal involvement and may result from rupture of a liver abscess in the left lobe of the liver into the pericardium or through extension of the right-sided pleural amebiasis.[16,28,29,34] It is estimated to occur in 3 percent of patients with hepatic abscesses.[29] It manifests as acute pericarditis with tamponade and, occasionally, as pneumopericardium.[28] Amebic liver abscess in the left lobe also may rupture directly into the left side of the chest.[65]

Cerebral amebic abscesses are seen only in individuals with amebic liver abscess and were found in 8 percent of patients with liver abscess discovered at autopsy in one study.[60] In other studies, lower rates of 0.66 to 4.7 percent of patients with amebic liver abscess having brain abscesses were reported.[48,98] Patients with cerebral amebiasis frequently are so ill from the intestinal, liver, and possibly lung involvement that neurologic signs are not always assessed easily. In 18 patients with proven cerebral amebiasis, initial neurologic examination was normal in 13 and only 1 patient later developed seizures.

Other foci of infection are rare findings, but amebic rectovesical fistula formation and involvement of pharynx, heart, aorta, and scapula have been reported. Cutaneous extension after the adherence of perforated, inflamed bowel to the skin is an extremely painful and rare complication.[16,75] This situation also may arise after invasion of the skin occurs from trophozoites emerging out from the rectum.[69]

DIAGNOSIS

The prevalence of diarrhea due to *E. histolytica* in the first year of life in impoverished children in the developing world may be as high as 10 percent.[71] A heightened suspicion of amebiasis should be present if the patient has been in a developing country as a resident or traveler. The diagnosis of amebiasis should be considered in any child with risk factors who has diarrhea, bloody stools, or stools with mucus; with a hepatic abscess; and/or with right upper quadrant pain, abdominal distention, or tachypnea.[56,70] In a patient with diarrhea, if blood is present in the stool (grossly bloody or occult blood positive), infectious (Shiga toxin–producing *E. coli*, *Salmonella*, *Shigella*, *Campylobacter*, and *E. histolytica*) and noninfectious (inflammatory bowel disease, diverticulosis, arteriovenous malformations, cancer) causes should be considered.

Serologic Tests

Serologic tests for anti-amebic antibodies also are a very useful tool in diagnosis, with sensitivity of 70 to 80 percent early in disease and approaching 100 percent sensitivity on convalescence.[97,100] The combined use of serology and stool antigen detection or PCR analysis offers the best diagnostic approach.

Microscopic Examination of Stool

Before the development of new antigen detection tests and PCR analysis, amebiasis was diagnosed by examining a stool sample through a microscope to determine whether *E. histolytica* cysts were present. This method often requires more than one specimen, however, because the number of cysts in the stool varies greatly. In addition, stool microscopy has limited sensitivity and specificity. The body's own immune system produces macrophage cells that can look like the amebae. Four different amebae—*E. histolytica*, which causes amebiasis, *E. dispar*, which does not cause disease, and *E. moshkovskii* and *E. bangladeshi*, which may cause diarrhea—look identical under a microscope.[22,91,95]

Noninvasive Diagnosis of Extraintestinal Amebiasis

Amebiasis outside the intestine has been even more difficult to diagnose. Clinical manifestations of extraintestinal disease vary widely, and less than 10 percent of individuals with amebic liver abscesses have identifiable *E. histolytica* in their stools. The TechLab *E. histolytica* II test, which differentiates the true pathogen *E. histolytica* from *E. dispar*, was reported to detect Gal/GalNAc lectin in the sera of 22 of 23 (96%) patients with amebic liver abscess tested before treatment with the anti-amebic drug metronidazole and 0 of 70 (0%) controls. After 1 week of treatment with metronidazole, more than 80 percent of patients became serum lectin antigen negative. Detection of *E. histolytica* Gal/GalNAc lectin in the sera using the TechLab *E. histolytica* II kit is sensitive to diagnose hepatic and intestinal amebiasis before the institution of metronidazole treatment.[41] A real-time PCR assay detected *E. histolytica* DNA in 49, 77, and 69 percent of blood, urine, and saliva specimens, respectively, from the amebic liver abscess patients.[40]

Noninvasive diagnostic procedures such as ultrasound, CT, and MRI can detect extracolonic amebiasis in the liver, paracecal masses, brain, and other sites, but they cannot distinguish between abscesses caused by amebae and those caused by bacteria, hampering proper treatment of the condition. Most patients with amebic liver abscess have a single abscess in the right lobe of the liver, although multiple lesions also can occur.[4] Chest radiographs show elevation of the right diaphragm in 56 percent of patients with hepatic abscess.[3] The diagnosis of cerebral amebiasis requires careful neurologic evaluation and radiographic evaluation with either CT or MRI.[16,48,60] In one case, *E. histolytica* DNA was detected by PCR in CNS to make the diagnosis.[98] Because of the risk for perforation, barium studies are relatively contraindicated in patients with amebic colitis.

Biopsy Studies

The colonic and rectal mucosa in amebic colitis usually reveals ulcerations with a diameter of 1 to 10 mm. Amebic trophozoites often are at the periphery of these necrotic areas, which can be sampled through a biopsy specimen taken during sigmoidoscopy or colonoscopy.[45,51] Because of the potential for perforation, colonoscopy should be undertaken with caution.

In patients with amebic liver abscesses, amebic trophozoites are found near the capsule of the abscess. Until more recently, the most accurate diagnostic test involved the examination by microscopy of a sample collected from the abscess tissue by needle aspiration, a procedure that is relatively insensitive, identifying amebic trophozoites only 20 percent of the time. PCR in contrast is a sensitive and specific means to identify *E. histolytica* in liver abscess material.[40]

DIFFERENTIAL DIAGNOSIS

Invasive amebic colitis may resemble ulcerative colitis, Crohn disease of the colon (inflammatory bowel disease), bacillary dysentery, or tuberculous colitis.[11,18,45,93] Stool *E. histolytica* antigen or PCR analysis, colonoscopic examination with biopsies, and serologic examination should be able to differentiate amebic colitis from these diseases. Histologic examination of involved colonic mucosa should differentiate amebic colitis, with its lack of inflammation and rare granulation tissue, from the inflammatory responses seen in ulcerative colitis, bacillary dysentery, and Crohn disease of the colon. Tuberculous colitis and Crohn disease are more likely to show granuloma formation than amebiasis. Ileocecal or small bowel involvement as seen on barium studies would suggest Crohn disease or tuberculosis of the gastrointestinal tract, rather than amebiasis. Tuberculous colitis usually is associated with pulmonary tuberculosis and with a strong reaction to tuberculin skin testing. In some cases, differentiating between invasive amebic colitis and inflammatory bowel disease may be impossible. If a patient with this differential diagnosis is placed on corticosteroids and his or her condition deteriorates, the corticosteroids should be stopped and repeat investigation for amebiasis should be performed.[18,70,75]

Amebic liver abscess must be differentiated from pyogenic abscesses and neoplastic lesions. Detection of *E. histolytica* Gal/GalNAc lectin in the sera using the TechLab *E. histolytica* II kit was helpful in one study to diagnose hepatic and intestinal amebiasis before the institution of metronidazole treatment.[41] Total leukocyte counts and cultures of blood may help to differentiate pyogenic and amebic abscesses. Many children with pyogenic liver abscesses have negative blood cultures, however. Often, amebic and pyogenic liver abscesses show similar features on CT and MRI. Occasionally, nuclear imaging with gallium is helpful because, in contrast to a pyogenic abscess, very few neutrophils are contained within an amebic liver abscess.[88,90] Gallium scanning of an amebic liver abscess may reveal a cold spot, possibly with a bright rim. For an individual with risk factors for amebiasis, several investigators recommend instituting treatment for amebic abscess for 3 or 4 days while serologic, antigen detection, or PCR results are awaited.[40,70] Patients with amebic liver abscess should respond to treatment in this length of time by becoming afebrile. No change in size of the liver or size of the abscess should be noted at this time because resolution of the abscess usually takes 2 months to several years.[4,82,83,94,105]

COMPLICATIONS

Complications of amebiasis may be prevented by early establishment of diagnosis and initiation of treatment with appropriate agents.[48,70] When complications occur, the prognosis generally is worse.

Invasive intestinal amebiasis has been associated most commonly with perforation and peritonitis,* which apparently are an end result of "necrotizing" or "toxic" amebic colitis. In children, perforation may be heralded by the appearance of an acute abdomen or pneumoperitoneum, with rapid progression to death, presumably from sepsis.[8,70,104] Surgical resection and therapy for endotoxic shock improve the prognosis.[104] This complication is not rare and accounts for more than 30 percent of deaths from amebiasis in children.[11,52] Massive intestinal hemorrhage causes approximately 3 percent of deaths from amebiasis. Multiple colonic strictures also can occur and cause obstructive symptoms. Fistulas to other organs or to the skin may develop.

Liver abscesses are an unusual manifestation of amebiasis in children, but their resultant complications account for approximately 40 percent of all deaths from amebiasis.[52] Liver abscess also was found in 13 percent of patients with amebiasis at postmortem examinations. Liver abscess with rupture into the abdomen was present in 8 percent of patients who died with amebiasis, and rupture of a liver abscess into the right pleural space was found in 12 percent.[52] Many patients with amebic liver abscess also have anaerobic bacteria in the abscess fluid.[86]

*References 8, 10, 50, 70, 96, 104.

In cases free of bacterial contamination, the fluid has few inflammatory cells and an acidic pH. Amebic pericarditis or pneumopericardium occurs rarely and is found in only 1 percent of patients whose deaths were caused by amebiasis.[28,29,34,52] The fluid is similar to that found in the pleural space. A cerebral abscess was found in 4 percent of patients with amebiasis who died.[52] It has been reported in fewer than 10 children, only 1 of whom survived.[9,16,48,60] Other complications include infections of the retroperitoneal space, stomach, spleen, esophagus, and duodenum.[60]

TREATMENT

Intestinal Amebiasis

Asymptomatic Intraluminal Amebiasis

Therapy for asymptomatic colonization differs from therapy for invasive infection. Asymptomatic infections may be treated with intraluminal agents, such as paromomycin or diloxanide furoate. Each agent has a high rate of success for eradication of cyst passage.[67,68] Paromomycin is a nonabsorbable aminoglycoside that is active against the cyst and trophozoite stages. High cure rates have been reported with a 7-day oral dose of paromomycin at 25 to 35 mg/kg/day in three divided doses (Table 209-2). Diloxanide furoate (Furamide) is a poorly absorbed agent that is quite active against only intraluminal amebiasis.[64] Cure rates have been greater than 90 percent with a 10-day oral course of diloxanide furoate at 20 mg/kg/day in three divided doses (maximum, 1500 mg/day).[67,68,76]

Acute Amebic Colitis

Nitroimidazoles, particularly metronidazole, are the mainstay of therapy for invasive amebiasis.[39] The oral dosage of metronidazole is 35 to 50 mg/kg/day (maximum, 2250 mg/day) in three divided doses for 7 to 10 days for severe intestinal or extraintestinal amebiasis. Metronidazole is concentrated in the ameba, probably via reduction of its nitro group by ferredoxin or flavodoxin-like electron transport proteins, which maintain a gradient for the entry of the unchanged drug. Metabolic intermediates of metronidazole damage DNA and possibly other macromolecules, and they deprive the organism of reducing equivalents by acting as an electron sink. Nitroimidazoles with longer half-lives (tinidazole, secnidazole, and ornidazole) are better tolerated and allow shorter periods of treatment.[41] For children 3 years of age or older, the oral dosage of tinidazole is 50 mg/kg/day (to a maximum of 2000 mg/day) for 5 days for severe intestinal or extraintestinal amebiasis (see Table 209-2).

Approximately 90 percent of patients who present with mild-to-moderate amebic dysentery have a response to nitroimidazole therapy. In the rare case of fulminant amebic colitis, adding broad-spectrum antibiotics to treat intestinal bacteria that may spill into the peritoneum is prudent; surgical intervention occasionally is required for acute abdomen, gastrointestinal bleeding, or toxic megacolon.[39] Agents such as metronidazole that are active against invasive and extraintestinal amebiasis are well absorbed and do not stay in the lumen long enough to have an effect on intestinal amebiasis. Parasites persist in the intestine in 40 to 60 percent of patients who receive nitroimidazole. Nitroimidazole treatment should be followed with paromomycin or the second-line agent diloxanide furoate to cure luminal infection.[41] Metronidazole and paromomycin should not be given at the same time because the diarrhea that is a common side effect of paromomycin may render assessing the patient's response to therapy difficult.[52-54]

Extraintestinal Amebiasis

Amebic Liver Abscess and Metastatic Amebiasis

Extraintestinal and severe intestinal amebiasis must be treated with the tissue-active agents. Metronidazole 35 to 50 mg/kg/day in three divided doses for 7 to 10 days is preferred because it is effective and relatively free of serious side effects (see Table 209-2).[2,3,64,88,90] It is effective for extraintestinal amebiasis in any location, although amebic brain abscesses usually are not treated successfully with any medications. Most patients with amebic liver abscess respond to metronidazole within 72 hours. For amebic colitis, follow-up therapy with a luminal agent is very important because of the high rates of asymptomatic intestinal colonization in patients with amebic liver abscess.

Therapeutic aspiration of an amebic liver abscess occasionally is required as an adjunct to antiparasitic therapy. Drainage of the abscess should be considered in patients who have no clinical response to drug therapy within 5 to 7 days or patients with a high risk of experiencing rupture of the abscess, as defined by a cavity with

TABLE 209-2 Pediatric Dosage of Drugs for Amebiasis

Type of Disease	Drug	Dosage
Asymptomatic colonization	Paromomycin	25-35 mg/kg/day in 3 doses × 7 days
Mild-to-moderate intestinal disease	Metronidazole	35-50 mg/kg/day in 3 doses × 7-10 days
	Tinidazole	50 mg/kg/day (maximum, 2000 mg) × 3 days
Severe intestinal and extraintestinal disease	Metronidazole	35-50 mg/kg/day in 3 doses × 7-10 days
	Tinidazole	50 mg/kg/day (maximum, 2000 mg) × 3 days

a diameter of more than 5 cm or by the presence of lesions in the left lobe.[103] Because many patients with amebic liver abscess also have anaerobic bacteria in the abscess fluid,[86] addition of antibiotics, drainage, or both to the treatment regimen in the absence of a prompt response to nitroimidazole therapy is reasonable. Image-guided percutaneous treatment (needle aspiration or catheter drainage) has replaced surgical intervention as the procedure of choice for reducing the size of an abscess.[103]

PROGNOSIS

Invasive disease develops in 50 million people each year, and 50,000 to 100,000 deaths result.[81,87,88] The case-fatality ratio is between 1 in 500 and 1 in 1000 diagnosed cases. Among patients with illness severe enough to require hospitalization, the case-fatality ratio is higher. One small study in children reported a 9 percent mortality rate and a 27 percent morbidity rate.[70]

Bowel necrosis or perforation is the cause of death from purely intestinal amebiasis, and early surgical intervention can reduce the mortality rate of these complications from 100 to 28 percent.[104] Amebic liver abscess has a case-fatality rate of 10 to 15 percent in combined figures of adults and children.[56,73,84] The mortality rate when pleural involvement is noted is 14 percent.[49,56] Amebic pericarditis has a case-fatality rate of 40 percent.[34] Cerebral amebiasis is fatal if untreated.[98]

FUTURE CONSIDERATIONS

Amebiasis would be prevented by eradicating fecal contamination of food and water. Providing sanitation and safe food and water for all children in developing countries is an important and achievable goal but will require massive societal changes and monetary investments. An effective amebiasis vaccine is a desirable and feasible goal to help the approximately 1 billion people living in unsanitary conditions. The high incidence of amebiasis in community-based developing country studies of infants, who bear the brunt of morbidity and mortality from diarrhea, suggests that an effective vaccine would improve child health.

The fact that humans naturally acquire partial immunity against intestinal infection indicates that barriers to stimulating an effective acquired immune response should not be insurmountable. Aiding vaccine design is the demonstration that several recombinant antigens, including the Gal/GalNAc-specific lectin, provide protection in animal models of amebiasis and that human immunity is linked to intestinal IgA against the lectin.[33,37,39] The high degree of sequence conservation of the Gal/GalNAc-specific lectin suggests that a vaccine could be broadly protective. Finally, the absence of epidemiologically significant animal reservoirs suggests that herd immunity could interrupt fecal-oral transmission in humans. The challenges will be to design vaccines capable of eliciting durable mucosal immunity, to understand the correlates of acquired immunity, and, most important, to enlist the continued support of industrialized nations to combat diarrheal diseases of children in developing countries.

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Species-Specific Immunity Induced by Infection with *Entamoeba histolytica* and *Entamoeba moshkovskii* in Mice

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Abstract

Entamoeba histolytica, the parasitic amoeba responsible for amoebiasis, causes approximately 100,000 deaths every year. There is currently no vaccine against this parasite. We have previously shown that intracecal inoculation of *E. histolytica* trophozoites leads to chronic and non-healing cecitis in mice. *Entamoeba moshkovskii*, a closely related amoeba, also causes diarrhea and other intestinal disorders in this model. Here, we investigated the effect of infection followed by drug-cure of these species on the induction of immunity against homologous or heterologous species challenge. Mice were infected with *E. histolytica* or *E. moshkovskii* and treated with metronidazole 14 days later. Re-challenge with *E. histolytica* or *E. moshkovskii* was conducted seven or 28 days following confirmation of the clearance of amoebae, and the degree of protection compared to non-exposed control mice was evaluated. We show that primary infection with these amoebae induces a species-specific immune response which protects against challenge with the homologous, but not a heterologous species. These findings pave the way, therefore, for the identification of novel amoebae antigens that may become the targets of vaccines and provide a useful platform to investigate host protective immunity to *Entamoeba* infections.

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Introduction

Amoebiasis, an infectious disease caused by the parasitic protozoan *Entamoeba histolytica* is responsible for over 50 million cases in tropical regions and nearly 100,000 deaths worldwide each year. Infection is initiated through the ingestion of cysts in contaminated food or water. *E. histolytica* primarily infects the intestine, and may cause a wide range of symptoms from mild diarrhea to serious dysentery. If untreated, the parasite can cause life-threatening hemorrhagic colitis and/or extra-intestinal abscesses [1-5].

E. histolytica trophozoites are able to colonize the human intestine by adhering to colonic mucins and subsequently to epithelial cells via cell surface lectin [6]. This lectin is important for colonic colonization by *E. histolytica*. A colonization-blocking vaccine targeting this parasite lectin could prevent trophozoite adherence and thus provide protection against subsequent invasive disease [7]. Furthermore, recently, it has also been reported that there is a correlation between the presence of anti-lectin fecal immunoglobulin A (IgA) antibodies and protection from parasitic colonization in humans and mice [7-9]. These reports suggest that amoebiasis can be controlled by acquired immunity.

Entamoeba moshkovskii is closely related to *Entamoeba dispar* and *E. histolytica* and is microscopically indistinguishable from them in its cyst and trophozoite forms [10]. Recently, we reported that *E. moshkovskii* causes diarrhea, colitis and weight loss in mice, and that in

Bangladeshi children, acquisition of *E. moshkovskii* infection was associated with diarrhea [11].

Here, using *E. histolytica* and *E. moshkovskii* infections in mice, we evaluate whether the immunity against reinfection that occurs following a primary infection is species-specific. We find that, following a primary infection with either *E. histolytica* or *E. moshkovskii*, mice are protected from re-challenge with a homologous species, but remained susceptible to a heterologous species. These results show, for the first time, that the immunity acquired during primary infection with *Entamoeba* spp. confers species-specific protective immunity.

Materials and Methods

Mice

Male CBA/J mice were purchased from Jackson Laboratories. Animals were maintained under specific pathogen free conditions at the Animal Research Center for Tropical Infectious Diseases, Nagasaki University, and were challenged when they were 5-8 weeks old. All experiments that involved mice were reviewed and approved by the Committee for Ethics on Animal Experiments of the Graduate School of Nagasaki University, and were conducted under the control of the Guidelines for Animal Experiments in the Graduate School of Medicine, Nagasaki University, and the Law (No. 105) and Notification (No. 6) of the Japanese Government pertaining to the use of experimental animals.

Parasite Culture and Infection

Trophozoites of *E. histolytica*, originally laboratory strain HM1:IMSS (American Type Culture Collection, Manassas, VA), were from Prof. Eric Houpt, University of Virginia, and were serially passaged *in vivo* through the ceca of mice [12]. 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, NIH, Bethesda, Maryland). Cecal contents were cultured at 37°C and 25°C, respectively, in BIS-33 medium supplemented with heat-inactivated 10% adult bovine serum, 25U/ml penicillin and 25 mg/ml streptomycin [13]. Trophozoites in the logarithmic growth phase were used in the experiments.

Intracecal inoculation of *Entamoeba* spp

Trophozoites of *E. histolytica* HM1:IMSS and *E. moshkovskii* Laredo strain were collected after incubating the tubes on ice for 5-10 minutes. Then, the number of trophozoites was counted. We anesthetized mice with Domitor (medetomidine hydrochloride: 0.1mg/kg) and Dormicum (midazolam: 0.1 mg/kg), shaved their abdomens to incise the skin, exteriorized each cecum from the peritoneum, and injected 150μl of 1×10^6 trophozoites into the 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 surgery. Survival rates were ≥90% in all mice.

Detection of each *Entamoeba* spp. by PCR using DNA extracted from stool of mice

For isolation of *Entamoeba* DNA from mouse stools, QIAamp DNA Stool Kits (QIAGEN, Valencia CA) were used according to manufacturer's instructions. The primer sequences used for PCR are as previously described [14].

Administration of metronidazole

For *in vivo* studies, stock solutions of metronidazole (Sigma Aldrich, St. Louis, MO) were prepared in 100% dimethyl sulfoxide at a concentration of 10 mg/mL and stored at 4°C. The stock solution was diluted 32 times with distilled water to 0.3125 mg/mL, in which the concentration of DMSO was 3.125%. Mice were treated orally with metronidazole at a dose of 12.5mg/kg of body weight. To cure primary infections with *E. histolytica* or with *E. moshkovskii*, all of mice challenged with *E. histolytica* or with *E. moshkovskii* were treated with 1 mL of metronidazole orally (0.3125 mg/mL) using gastric intubation on day 14 post-infection. Naïve mice were also administered with metronidazole and used as control.

Statistical analysis

Differences between groups were analyzed for statistical significance with unpaired Student's *t*-test and χ^2 test. All of these were performed using Excel software. Probabilities below 0.05 were considered statistically significant.

Results

E. *moshkovskii* infections were resolved earlier than *E. histolytica* infections

We have previously demonstrated that C3H/HeN, C3H/HeJ and CBA/J mice allow the establishment of *E. histolytica* and *E. moshkovskii* infections, while many strains of mice including C57BL/6 and BALB/c mice do not, indicating that susceptibility to *E. histolytica* and *E. moshkovskii* infection is dependent on the genetic background of the host [11,12,15-17]. Trophozoites of *E. histolytica* and *E. moshkovskii* were intracecally inoculated into CBA/J mice. As expected, both *E. histolytica* and *E. moshkovskii* succeeded in infecting CBA/J mice after challenge (Figure 1). *E. histolytica* infected the ceca in approximately 80% of CBA/J mice (16 of 20) as confirmed by both culture and PCR of intracecal contents two days after challenge. In contrast, *E. moshkovskii* infected the ceca of CBA/J mice in approximately 65% of mice (13 of 20) at the same point. At day 14 post-challenge, the infection rate of *E. histolytica* was approximately 60% (12 of 20 mice positive), though that of *E. moshkovskii* was approximately 5% of mice (1 of 20). At 21 days post-challenge, the infection rate of *E. histolytica* was approximately 58% (11 of 19) and that of *E. moshkovskii* was 0%.

Metronidazole Sensitivity in E. *moshkovskii*

So as to treat mice infected with *E. histolytica* and *E. moshkovskii*, the effect of metronidazole on the growth and survival of *E. histolytica* and *E. moshkovskii* trophozoites was evaluated *in vitro*. The number of viable cells in glass tubes

Figure. 1

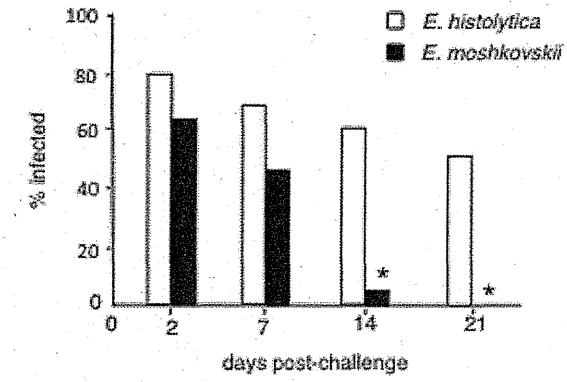

Figure 1. Entamoeba moshkovskii infections were resolved earlier than *Entamoeba histolytica* infections. CBA/J mice were intracaecally infected with 1 × 10^6 trophozoites of *E. histolytica* and *E. moshkovskii*. Infection rate was monitored by detecting amoebae in caecal content and by amplifying the amoeba gene from faecal DNA on days 2, 7, 14, and 21. Infection rate of mice with *E. histolytica* and *E. moshkovskii* was shown as open and closed columns, respectively. Values show the representative result out of 3 individual experiments. Asterisks indicate statistical significance with $p < 0.05$ between mice infected with *E. histolytica* and *E. moshkovskii* by χ^2 test.

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Figure 2

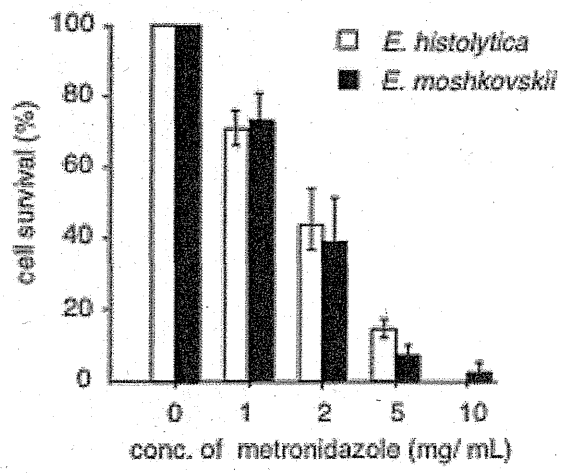

Figure 2. The effect of metronidazole on the growth and survival of *Entamoeba histolytica* and *Entamoeba moshkovskii* trophozoites was evaluated *in vitro*. *E. histolytica* and *E. moshkovskii* were incubated with various concentrations of metronidazole for 48h. Then, the number of viable cells was counted and the proportion of it versus initial number was shown as open and closed columns, respectively.

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was counted after incubation of *E. histolytica* and *E. moshkovskii* with various concentrations of metronidazole for 48h. The numbers of *E. histolytica* and *E. moshkovskii* treated with metronidazole decreased significantly in a dose dependent manner (Figure 2).

Amoebic infection induced species-specific protective immunity

In order to examine whether protection against re-infection can be induced by primary infection, the mice that allowed the establishment of the primary infection with *E. histolytica* or *E.*

Figure 3

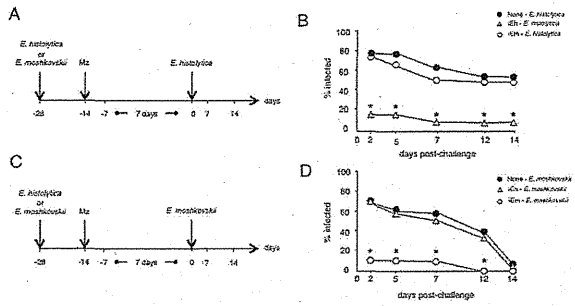

Figure 3. Amoebic infection induced species-specific protective immunity. Mice were infected with 1 × 10^6 trophozoites of *Entamoeba histolytica* (open triangle) or *Entamoeba moshkovskii* (open circle) and treated with metronidazole (Mz) on day 14 following induction of the primary infection. The clearance of amoeba was confirmed seven days after treatment by PCR. Mice were kept without any intervention for an additional week, and then re-challenged with 1 × 10^6 trophozoites of *E. histolytica* (A, B) or *E. moshkovskii* (C, D) at 14 days after treatment. The number of mice used was as follows: for naïve→*E. histolytica*, N=16: for *E. histolytica*→*E. histolytica*, N=20: for *E. moshkovskii* →*E. histolytica*, N=20 (A, B); for naïve→*E. moshkovskii*, N=20: for *E. histolytica*→*E. moshkovskii*, N=20: for *E. moshkovskii* →*E. moshkovskii*, N=20 (C, D). Asterisks indicate statistical significance with $p < 0.05$ by χ^2 test between mice infected with *E. histolytica* and *E. moshkovskii* in the primary infection.

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moshkovskii were treated with metronidazole on day 14 and used for the secondary challenge. The clearance of amoeba was confirmed seven days after treatment by PCR. Mice were kept without any intervention for an additional week, and then re-challenged with *E. histolytica* or *E. moshkovskii* a total of 14 days after treatment (Figure 3A, C). The mice infected with *E. histolytica* and treated with metronidazole showed resistance to homologous re-challenge infection (Figure 3B), but allowed establishment of infection with the heterologous species *E. moshkovskii* in a manner similar to that seen in naive mice (Figure 3D). Similarly, mice infected with *E. moshkovskii* and treated with metronidazole showed resistance to homologous re-challenge infection with *E. moshkovskii* (Figure 3D), but allowed the establishment of infection with the heterologous species *E. histolytica* (Figure 3B). Thus, mice that experienced primary amoebic infection acquired resistance to secondary homologous species infection. However, primary amoebic infection did not confer protection against heterologous species secondary infection. These results show that intestinal amoebic infection induces species-specific protective immunity.

The protection induced by primary infection lasts more than four weeks

To examine how long the protection observed against secondary infection lasts, mice were re-challenged with homologous or heterologous amoebae on day 35 after treatment with metronidazole 14 days after primary infection. The clearance of amoeba was confirmed seven days after treatment by PCR (Figure 4A, C). As shown in Figure 4B and 4D, 35 days after the treatment of the primary infection, mice were resistant to homologous re-challenge, but were susceptible to heterologous species infection. Mice kept for 35 days after the treatment of the primary infection showed increased infection rates compared to those kept just for 14 days, a phenomenon that was most apparent on day 2 post re-challenge in the case of *E. histolytica* and on days 2 and 5 post challenge with *E. moshkovskii* (Figure 4B and 4D). These results suggest that the protection induced by primary infection may include not only memory responses but also remaining primary immune responses, both of which are species specific.

Infection-induced species-specific immunity protects mice from weight loss

During the primary infection, mice infected with *E. moshkovskii* suffered severe symptoms. Following re-challenge with *E. moshkovskii* (homologous species) 14 days after treatment of the primary infection, mice did not show any weight loss (Figure 5A). Slight weight loss was observed, however, in mice re-challenged 35 days after treatment, but the severity of weight loss was much smaller than that observed during the primary infection (Figure 5B). The weight loss was also ameliorated in mice re-infected with *E. histolytica*, when having been given a primary infection with the homologous species (data not shown).

Discussion

Our results clearly demonstrate that exposure to, and subsequent drug clearance of, the parasitic amoebae *E. histolytica* and *E. moshkovskii* invokes a strong immune response that protects mice from subsequent infection with a homologous species. This protection is species specific, affording little to no protection against a heterologous species challenge. The fact that this strong species-specific immunity was shown to last at least 35 days following the treatment of the initial infection suggests that memory responses are involved.

Figure 4

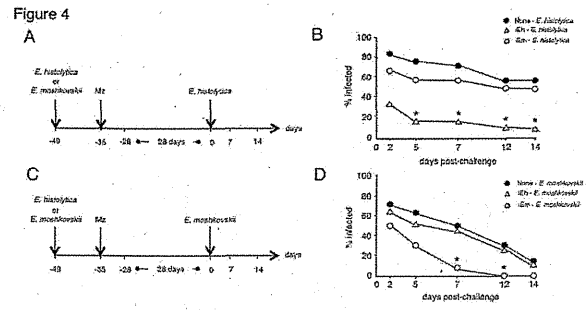

Figure 4. The protection induced by primary infection lasts more than four weeks. Mice were infected with 1 × 10^6 trophozoites of *Entamoeba histolytica* (open triangle) or *Entamoeba moshkovskii* (open circle) and treated with metronidazole (Mz) on day 14 following induction of the primary infection. The clearance of amoeba was confirmed seven days after treatment by PCR. Mice were kept without any intervention for 28 days, and re-challenged with 1 × 10^6 trophozoites of *E. histolytica* (A, B) or *E. moshkovskii* (C, D) at 35 days after treatment. The number of mice used was as follows: for naïve→*E. histolytica*, N=16: for *E. histolytica*→*E. histolytica*, N=20: for *E. moshkovskii* →*E. histolytica*, N=26 (A, B); for naïve→*E. moshkovskii*, N=15: for *E. histolytica*→*E. moshkovskii*, N=20: for *E. moshkovskii* →*E. moshkovskii*, N=20 (C, D). Asterisks indicate statistical significance with $p < 0.05$ by χ^2 test between mice infected with *E. histolytica* and *E. moshkovskii* in the primary infection.

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Figure 5

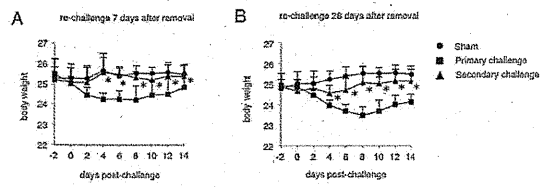

Figure 5. The change of body weight was monitored after re-challenging mice with *Entamoeba moshkovskii*. The naïve mice or mice exposed to primary *E. moshkovskii* infection were re-challenged with 1 × 10^6 trophozoites of *E. moshkovskii* at 7 or 28 days after confirming the clearance of primary infection. The studies were repeated 3 times with similar results. Asterisks indicate statistical significance with $p < 0.05$ between the groups of mice with primary and secondary infection using unpaired Student's *t*-test.

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Despite the fact that it has long been suspected that people may acquire immunity against amoebae, as older children in endemic areas are infected less frequently than younger children [18], this work constitutes the first experimental proof of this phenomenon. The molecular and cellular mechanisms responsible for the observed protection have not been addressed in this work. Mucosal IgA has been reported to be associated with protection against intestinal amoebiasis in humans, mice and baboons [7,8,19]. Indeed, monoclonal IgA specific for the *E. histolytica* galactose inhibitable adherence (GalNAc) lectin heavy subunit (HgL) is thought to inhibit its interaction with a host sugar moiety in colonic mucins, resulting in the failure of amoebae to settle within the intestines [20]. Furthermore, we and Guo et al. recently reported that IFN-γ derived from amoeba-specific T cells plays a protective role against *E. moshkovskii* (unpublished data) and *E. histolytica* [21], respectively, suggesting that T cells as well as antibodies specific for amoebic antigens are involved in acquired resistance to intestinal amoebic infections.

The phenomenon of species-specific immunity against parasitic pathogens has been studied in a number of parasitic species, perhaps most comprehensively with the *Plasmodium* species responsible for malaria [22]. For this pathogen, which exhibits both species and strain specific immunity, antigenic variation of major parasite surface antigens such as the merozoite surface protein 1 (MSP1), induces antibody-mediated immune responses that are effective only against the inducing-strain [22,23]. Such highly polymorphic strain- and species-specific antigens are thought to evolve through the actions of positive diversifying selection, so that proteins that are targeted by the host immune response rapidly accumulate polymorphisms. Here we show, for the first time, that the phenomenon of species-specific immunity also exists for *Entamoeba spp*. It seems probable that this is due to

polymorphisms in major antigen target proteins between species. If so, then such antigens may be identified by comparative genomics. Of particular interest are the GalNAc-lectin HgL proteins previously implicated in antibody-mediated protection against *E. histolytica*. Nucleotide sequence comparisons of the genes encoding this protein in *E histolytica* and *E. moshkovskii* may shed further light on this.

We found that *E. moshkovskii* is susceptible to the anti-amoeba drug metronidazole both *in vitro* and *in vivo* to the same degree as *E. histolytica*. This finding supports the use of this drug in the treatment of pathogenic *E. moshkovskii*, and may ease concern of treatment failure following cases of misdiagnosis of *E. moshkovskii* as *E. histolytica*.

In summary, we show that exposure to a single drug cured amoebic infection confers resistance to re-challenge with the homologous, but not a heterologous species, for the first time, in which species-specific acquired immunity has been demonstrated for amoebic infections. This work paves the way, therefore, for the identification of novel amoebae antigens that may become the targets of vaccines.

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Author Contributions

Conceived and designed the experiments: CS SH. Performed the experiments: CS TI KS MH TT SK. Analyzed the data: CS RC HH SH. Contributed reagents/materials/analysis tools: CS SK SH. Wrote the manuscript: CS RC SH.

Assessment of Real-Time Polymerase Chain Reaction Detection of *Acanthamoeba* and Prognosis Determinants of *Acanthamoeba* Keratitis

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Objective: To evaluate the diagnostic value of real-time polymerase chain reaction (PCR) for detecting *Acanthamoeba* in eyes diagnosed with *Acanthamoeba* keratitis (AK) by conventional tests. In addition, to determine the preoperative prognosis-determining factors in eyes with AK.

Design: Retrospective, cross-sectional study.

Participants: A total of 104 eyes of 103 patients who were diagnosed with AK or with bacterial or bacteria-associated keratitis (BK) by conventional tests.

Methods: Twenty-nine eyes with AK and 75 eyes with BK were evaluated for *Acanthamoeba* and bacterial DNA by real-time PCR. The *Acanthamoeba* copy numbers, bacterial load, and clinical parameters in the patients with AK were assessed for those significantly associated with poor outcome, that is, final visual acuity of <20/50 or requiring keratoplasty, by logistic regression analysis.

Main Outcome Measures: Acanthamoeba DNA copy number, bacterial DNA copy number, and odds ratio (OR) for poor prognosis.

Results: The detection of amoebic DNA was 50 times more sensitive by real-time PCR than by conventional cyst counting. The *Acanthamoeba* copy numbers at the first visit (mean: $4.7\times10^5\pm3.2\times10^5$ copies) were significantly correlated with the AK stage, and both were significant risk factors for a poor outcome. The *Acanthamoeba* DNA copy numbers at the first visit and AK stage had a significantly high risk for poor outcome (OR of *Acanthamoeba* DNA copy per logarithm of copy numbers: 3.48, 95% confidence interval [CI], 1.04–111.63, $P<0.05$; OR of AK stage: 2.8 per stage increase, 95% CI, 1.07–7.30, $P<0.05$, after adjustment of age). In the AK cases with poor outcome, the amoebic DNA was not reduced by more than 90% after 1 month of treatment. The weak amoebic reduction was significantly associated with advanced AK stages or previous use of steroids. Bacterial 16S rDNA was detected in 53.6% of the eyes with AK, but it was not associated with any risk for refractoriness.

Conclusions: Real-time PCR was effective in detecting and managing AK. The *Acanthamoeba* copy number and AK stage at the first visit were significantly associated with poor outcome.

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Acanthamoeba keratitis (AK) is a destructive disease process with significant visual morbidity, and prompt diagnosis is important for good visual outcome. However, the available *Acanthamoeba* tests are generally not sensitive enough for precise diagnosis. At present, staining corneal smears with Calcofluor and Fungiflora Y is probably the most effective method of diagnosing AK.[1] However, false negatives can occur, which cause a delay of treatment leading to poor visual outcomes.

Another difficulty with AK is in evaluating the effectiveness of a treatment protocol because of the absence of reliable and quantitative methods of determining the *Acanthamoeba* copy numbers. In refractory cases, the immunologic responses can worsen even after weeks of intensive treatment, and dense infiltrations can then develop in the lesion. Under these conditions, qualitative tests (e.g., culturing and smear staining) do not provide sufficient information on whether the treatment has reduced the *Acanthamoeba* copy numbers. Thus, tests that are more sensitive and provide quantitative values of the *Acanthamoeba* copy numbers will be helpful.

Acanthamoeba is innocuous unless embedded in a diseased cornea or central nervous system. In addition, *Acanthamoeba* can be an opportunistic pathogen in immune-compromised hosts.[2] Free-living *Acanthamoeba* ingest mainly bacteria, and AK can develop in eyes with bacterial keratitis and bacteria-associated keratitis (BK).

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There is also a possibility that AK can develop in eyes with advanced BK, but because of the lack of sensitive and quantitative tests for *Acanthamoeba*, little information is available on whether such a cause is possible. To evaluate such cases, it would be valuable to determine the level of the *Acanthamoeba* copy numbers relative to the bacterial load in the lesion.

Polymerase chain reaction (PCR) measurements are known to have high sensitivity in detecting amoebic DNA.[3–6] Real-time PCR has high sensitivity and the ability to obtain quantitative values of the degree of AK.

Thus, this study determines the sensitivity and specificity of real-time PCR in detecting *Acanthamoeba* DNA in eyes with AK and with BK. In addition, we determined whether the copy numbers of *Acanthamoeba* were correlated with the bacterial load in eyes diagnosed with AK and BK by standard clinical tests. We also used logistic analysis to determine the clinical parameters that were significantly associated with poor outcomes. We shall show that the stage of the AK and the *Acanthamoeba* copy numbers at the initial examination are significantly associated with poor visual outcomes.

Materials and Methods

Diagnosis of *Acanthamoeba* Keratitis and Treatment

A total of 104 eyes of 103 patients with suspected infectious keratitis including AK and BK were studied between January 2006 and December 2010. Of these 103 patients, 49 were men and 54 were women, with a mean age of 48.2±2.2 years. Twenty-nine eyes of 28 patients were diagnosed with AK. Eleven were men and 17 were women, with a mean age of 26.4±1.7 years. Twenty-seven of the patients with AK (96.4%) were contact lens wearers.

Seventeen healthy subjects (9 male and 8 female) with a mean age of 36.4±1.4 years, who were not contact lens wearers, were enrolled for examination of their conjunctival scraping by real-time PCR as normal controls.

The diagnosis of AK was based on a modification of a described method.[4,5] A definitive diagnosis of AK was based on the clinical characteristics and identification of one or more of the following findings in the laboratory tests:[1–4] (1) identification of trophozoites or cysts in corneal scrapings stained with Fungiflora Y,[1] (2) positive *Acanthamoeba* cultures, (3) pathologic identification of *Acanthamoeba* cysts on keratoplasty specimens, and (4) identification of *Acanthamoeba* genome from corneal scrapings by PCR.

To collect tissues for staining or culture, the lesions were scraped or ablated to obtain a sufficient amount of tissue to maximize the amount of *Acanthamoeba* trophozoites or cysts. After this, the corneal bed was swiped, and the swab was processed for *Acanthamoeba* DNA.

The stage of the AK was determined at the first visit and based on the clinical findings observed by slit-lamp biomicroscopy.[4] Briefly, AK was divided into 5 stages of disease severity: 1 = epitheliitis, 2 = epitheliitis with radial neuritis, 3 = anterior stromal disease, 4 = deep stromal keratitis, and 5 = ring infiltrate or extra corneal inflammation.

After a definitive diagnosis of AK, most of the patients were treated with hourly instillations of 0.2% fluconazole, 1% voriconazole, 0.02% polyhexamethylene biguanide, 0.02% chlorhexidine gluconate, and 200 mg of oral itraconazole. The corneas were debrided to reduce the *Acanthamoeba* load and facilitate drug penetration.

The diagnosis of BK was based on positive microbial identification in smear staining or culturing. Cases with positive microbiological results and responsive to appropriate antibiotics were defined as BK. Other cases that did not meet any of the criteria were classified as BK.

The study protocol was approved by the Tottori University Ethics Committee, and the procedures used conformed to the tenets of the Declaration of Helsinki. An informed consent was obtained from all of the participants after an explanation of the procedures to be used.

Real-Time Polymerase Chain Reaction

DNA was extracted from the scrapings of the corneal lesions with the QIAamp DNA mini kit (Qiagen, Hilden, Germany).[6] The 18S rDNA of *Acanthamoeba*, which distinguishes it from other amoeba genera (*Hartmannella, Naegleria, Balamuthia, Nuclearia,* and *Vahlkampfia*), was amplified using reported primers and probe sets.[3]

Forward: 5'-CGACCAGCGATTAGGAGACG-3'

Reverse: 5'-CCGACGCCAAGGACGAC-3'

TaqMan Probe: 5'-FAM-TGAATACAAAACACCACCATCG-GCGC-BHQ

Real-time PCR was performed and analyzed using the LightCycler (Roche, Basel, Switzerland) under the following conditions: 95°C for 15 minutes, followed by 50 cycles at 95°C for 0 seconds, and 60°C for 1 minute.

A standard curve was created using a dilution series with known amounts of genomic DNA from *Acanthamoeba castellanii* ATCC30010D. A detection of more than 1 copy was classified as *Acanthamoeba* DNA positive.

The total bacterial load was determined by real-time PCR using a broad-range (universal) probe and primers sets that detect the 16S rDNA from the domain *Bacteria*.[7]

Forward: 5'- TCCTACGGGAGGCAGCAGT-3'

Reverse: 5'- GGACTACCAGGGTATCTAATCCTGTT-3'

TaqMan Probe: 5'-FAM- CGTATTACCGCGGCTGCTG-GCAC-BHQ

The bacterial copy number (i.e., the total bacterial load) was calculated by a standard curve generated by using defined numbers of cloned templates.

Statistical Analyses

Data are presented as the mean ± standard error of the means. To evaluate the significance of the differences between groups, unpaired *t* tests or Mann–Whitney *U* tests were used. Spearman correlation analysis was used to determine the coefficients of correlation (ρ) between factors. Chi-square and Fisher exact tests were used to test the significance of the associations between the 2 kinds of classification. Multivariate logistic regression analysis was carried out to compute the odds ratios (ORs) and the 95% confidence intervals (CIs). In bilateral AK cases, the visually poorer eye was used for the statistical analyses. $P<0.05$ was considered significant.

Results

Sensitivity of Real-Time Polymerase Chain Reaction in Detecting *Acanthamoeba*

Although the detection of microbes by real-time PCR is known to be sensitive, the degree of sensitivity for *Acanthamoeba* has not

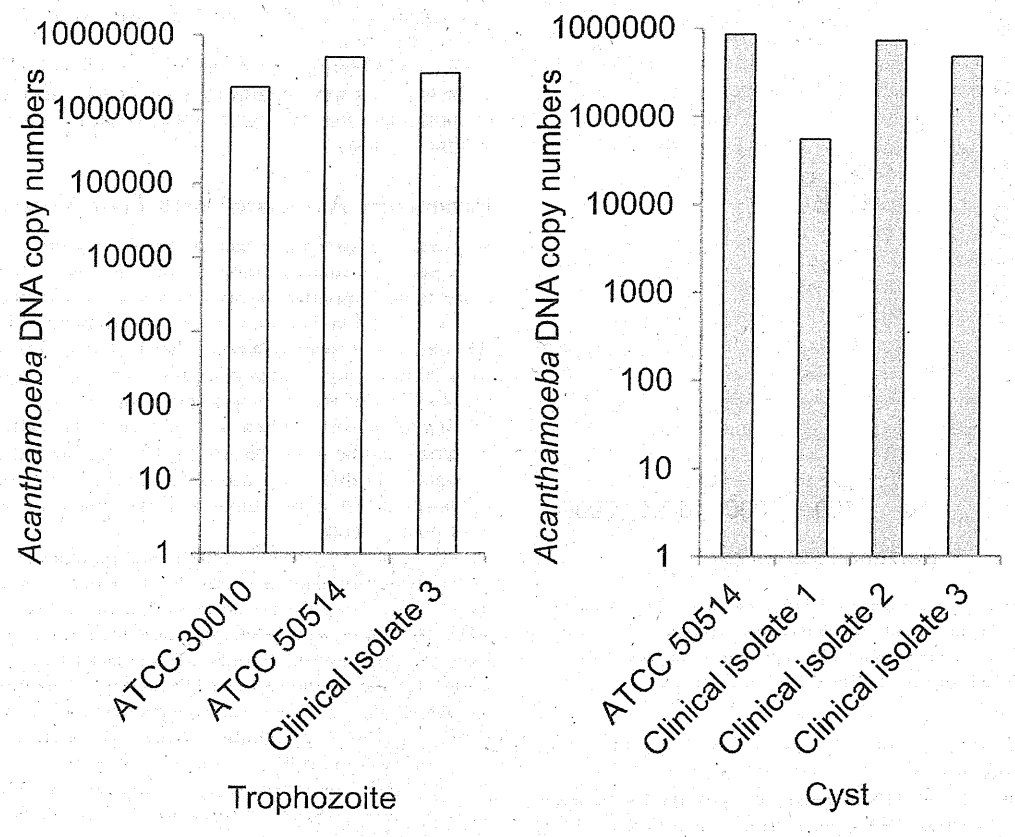

Figure 1. Evaluation of *Acanthamoeba* DNA detection in cultured *Acanthamoeba* cysts and trophozoites. Different strains of *Acanthamoebae* were cultured, and their cysts or trophozoites were diluted to 10 000 counts/ml. The extracted DNA was assessed for the *Acanthamoeba* DNA copy numbers. *Acanthamoeba* DNA detection by real-time polymerase chain reaction had better sensitivity for detecting both cysts and trophozoites. ATCC = American Type Culture Collection.

been determined. Generally, conventional microbiological tests rely on counting the number of amoebic bodies visually. Therefore, we first assessed how many copies of amoebic DNA can be detected for a known number of *Acanthamoeba* trophozoites or cysts (Fig 1). Clinical isolates and American Type Culture Collection strains were used as reference *Acanthamoeba*, and the trophozoites or cysts were diluted to 10 000 counts/ml in suspension.

Amoebic DNA was extracted from the suspension and assessed for the copy numbers using real-time PCR. Our results showed that the copy number detected by real-time PCR had approximately 300 times more sensitivity than visually counting trophozoites and 50 times more sensitivity than counting cysts (Fig 1).

To evaluate the specificity of *Acanthamoeba* real-time PCR in normal eyes, we also examined conjunctival scraping from 17 normal healthy subjects who were not contact lens wearers. No *Acanthamoeba* DNA (<1 copy) was detected in any of the scrapings.

Diagnostic Value of *Acanthamoeba* Real-Time Polymerase Chain Reaction

Acanthamoeba DNA was detected in 25 of the 29 AK eyes (86.2 %) by real-time PCR. The mean *Acanthamoeba* copy number was $4.7\times10^5\pm3.2\times10^5$ copies. The rate of detecting *Acanthamoeba* by smear staining with Fungiflora Y, which we have reported to be sensitive for detecting *Acanthamoeba*, was examined.[1] Among the 29 AK eyes, 26 underwent a Fungiflora test on diagnosis, and 22 eye samples were cultured. With Fungiflora Y staining, *Acanthamoeba* cysts were detected in 20 of 26 eyes (76.9%). In contrast, cultures of corneal specimens were positive in 12 eyes of 22 AK eyes (54.5%).

Acanthamoeba keratitis is generally associated with bacterial infection, but the specificity of identifying *Acanthamoeba* by real-time PCR has not been thoroughly evaluated in corneas diagnosed with BK. Therefore, we determined whether *Acanthamoeba* can be detected in BK cases using real-time PCR and Fungiflora Y staining. In 75 BK cases, none of the eyes was positive for *Acanthamoeba* DNA. Thus, the specificity of misdiagnosing AK by real-time PCR in eyes with BK was 100%.

When Fungiflora Y staining was assessed for specificity using 39 cases of BK, *Acanthamoeba* cyst-like staining was detected in 1 of 39 of the eyes with BK. This positive case was a contact lens user with *Pseudomonas aeruginosa* keratitis. Thus, the specificity of Fungiflora staining for diagnosing AK and not BK was 97.5%.

Evaluation of Bacterial Load by Real-Time Polymerase Chain Reaction

We next evaluated the bacterial load in the corneal lesions of eyes diagnosed as BK at their first visit or as a referral to Tottori University Hospital. For this, we used universal primers and real-time PCR for quantification of the bacterial DNA load. Bacterial

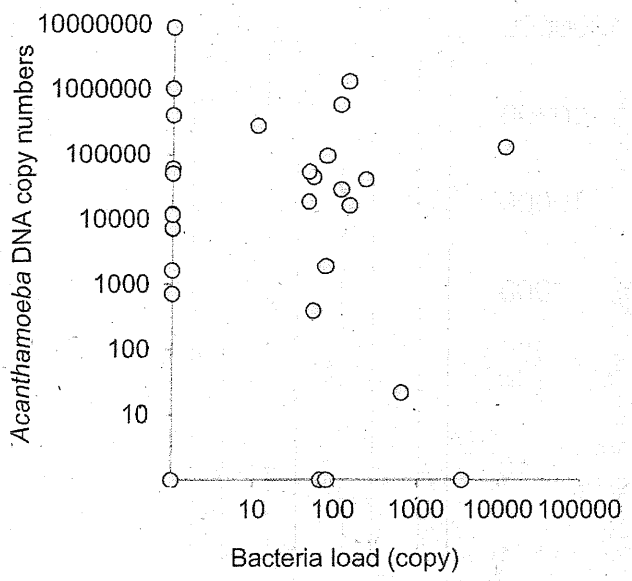

Figure 2. Evaluation of *Acanthamoeba* DNA copy number in *Acanthamoeba* keratitis determined by real-time polymerase chain reaction in relation to bacterial load. *Acanthamoeba* DNA copy number at first visit is not significantly correlated with the bacterial load in the lesion.

DNA was detected in 55 of 75 eyes (73.3%), and the mean bacterial DNA load was $8.8\times10^4\pm4.0\times10^4$ copies.

In the AK cases, 53.6% (15/28 eyes) were positive for bacterial DNA. The mean bacterial DNA copy number was $5.8\times10^2\pm4.0\times10^2$ copies. As expected, this was significantly lower than that in the BK eyes ($P<0.005$) by 10^2-fold.

To determine whether the development of AK was significantly associated with the bacterial load, we used Spearman correlation analysis to determine the relationship between the amoebic DNA copy number and the bacterial load in eyes with AK at the first visit. Amoebic DNA appeared inversely correlated with bacterial load (Fig 2); however, this was not statistically significant (Fig 2).

Correlation of *Acanthamoeba* DNA Copy Number and Stage of *Acanthamoeba* keratitis and Visual Acuity at First Visit

We next determined whether the stage of the AK was significantly associated with the copy number of *Acanthamoeba*. The *Acanthamoeba* copy number in the corneal specimens at the first visit was classified into 5 groups: 0 = not detected, 1 =≤1000, 2 =>1000 but ≤10 000, 3 =>10 000 but ≤100 000, and 4 =>100 000 copies. The relationship between the copy numbers of *Acanthamoeba* for each corneal specimen at the first visit to the stage of the AK was determined by Spearman correlation analysis. The stage of the AK was significantly correlated with the *Acanthamoeba* copy number ($\rho=0.53$, $P<0.05$; Fig 3A). In addition, the Acanthamoeba copy number was significantly correlated with the visual acuity in logarithm of the minimum angle of resolution units at the first visit ($\rho=0.37$, $P<0.05$; Fig 3B).

Next, the bacterial load was divided into 5 groups (0 = not detected, 1 =≤10, 2 =>10 but ≤100, 3 =>100 but ≤1000, 4 =>1000 but ≤10 000, and 5 =>10 000 copies). *Acanthamoeba* keratitis cases in the early stages were not significantly associated with the bacterial load (Fig 3C), and the correlation between the visual acuity and the bacterial load was not significant (Fig 3D). Thus, the *Acanthamoeba* copy number but not bacterial load was significantly associated with the disease severity and visual acuity before treatment.

We also determined whether the *Acanthamoeba* copy numbers at the first visit were significantly associated with the use of steroid or contact lens use. No significant associations were found (Mann–Whitney *U* test).

Parameters Associated with Poor Visual Outcome

We sought to determine factors that were significantly associated with poorer visual outcome in the AK cases after treatment of more than 2 months. A poor outcome was defined as a visual acuity <20/50 at the last visit or a requirement of keratoplasty. In AK cases with poor outcome, the *Acanthamoeba* copy numbers were 20 times higher than AK cases with good outcomes ($P<0.05$, Fig 4A). All of the cases with poor outcome had >10 000 copies of *Acanthamoeba*. When we evaluated the differences of the bacterial load between the AK cases with poor and good outcome, no significant difference was observed (Fig 4B). Thus, high *Acanthamoeba* DNA copy numbers at the first visit were associated with poor outcome.

We next evaluated the pretreatment parameters of the AK eyes that were significantly associated with poorer visual outcomes. By using logistic regression analysis, we calculated the risk of AK stage, *Acanthamoeba* DNA copy number at the first visit, bacterial load, previous use of steroids, and contact lens use (Table 1). We found that the *Acanthamoeba* DNA copy number and the stage of the AK at the first visit were the highest risk factors. The *Acanthamoeba* DNA copy numbers at the first visit had the highest risk for poor outcome (OR per category, 3.48; 95% CI, 1.04–111.63, $P<0.05$, after adjustment of age; Table 1). The AK stage had the second highest OR of 2.8 per stage increase (95% CI, 1.07–7.30, $P<0.05$, after adjustment of age). The previous use of steroids was not a significant risk (OR 8.84) for poor outcome ($P=0.07$). Other factors, including bacterial load and contact lens use, were not significant risk factors.

Parameters Associated with Unresponsive Reduction of *Acanthamoeba* Copy Numbers

Our findings indicate that the *Acanthamoeba* DNA copy number was significantly associated with the visual outcome. When we examined the *Acanthamoeba* copy number, all favorable outcome cases had a reduction in the copy number by >90% after 1 month of treatment (Fig 5A). In the cases with poor outcome, only 28.6% responded favorably to the treatment, and poor outcome was significantly associated with unresponsive reduction in the copy number.

To understand the cause of the outcome-related factors, an *Acanthamoeba* copy reduction was defined as a >90% reduction of *Acanthamoeba* copy numbers within 1 month of treatment. When the stage of the AK was compared between the responsive and unresponsive cases for *Acanthamoeba* copy reduction, the stage before the treatment of the unresponsive cases was significantly more advanced (Mann–Whitney *U* test, $P<0.01$, Fig 5B). The unresponsive *Acanthamoeba* copy reduction and previous steroid use were significantly associated ($P<0.05$, Fisher exact test). Five of the 23 unresponsive AK cases were previous steroid users, and the bacterial load in the unresponsive cases was not significantly associated with a decrease of *Acanthamoeba* copy numbers (unresponsive: 99±44 copies, responsive: 223±183 copies).

Finally, we determined which factors (e.g., the AK stage, amoebic DNA copy number, and bacterial load at the first visit) were significantly associated with an unresponsive DNA reduction. Logistic regression analysis was used to calculate the risks (Table 2). The AK stage was significantly associated with the DNA reduction with an OR of 8.00 per stage (95% CI, 1.06–

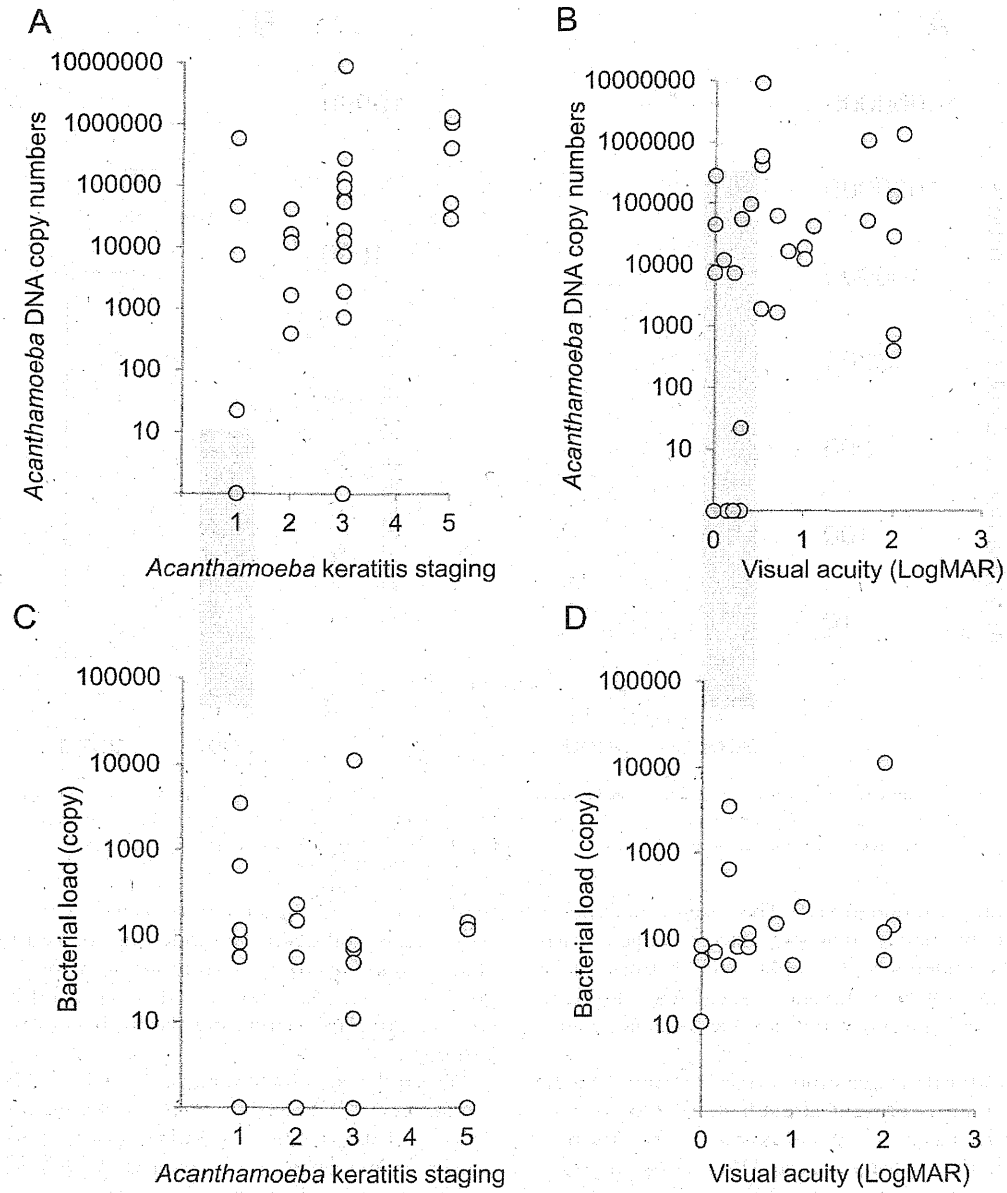

Figure 3. Significant correlation of *Acanthamoeba* DNA copy number with stage of *Acanthamoeba* keratitis (AK) and visual acuity. *Acanthamoeba* DNA copy number at first visit was significantly correlated with AK stage (ρ=0.53) **(A)** and logarithm of the minimum angle of resolution visual acuity (ρ=0.37) **(B)** by Spearman correlation analysis (P<0.05). Bacterial load in the lesion is not significantly correlated with the AK stage **(C)** or visual acuity **(D)**. logMAR = logarithm of the minimum angle of resolution.

58.82, P<0.05, after adjustment of age). The amoebic DNA copy number at the first visit also had a similar risk. The bacterial load at the first visit was not significantly associated with the DNA reduction.

Discussion

Acanthamoeba keratitis is a persistent infection and generally requires prolonged intensive treatment. However, our understanding of how to treat this disease remains undetermined. Presumably, the clinical presentation of AK (i.e., the stage of the AK) reflects the *Acanthamoeba* load, and the immunologic responses significantly affect its prognosis. Culturing, smear staining, and confocal microscopy are widely used for diagnosing AK. However, their qualitative nature and low sensitivity limit the amount of information that can be obtained to determine the cause of the AK. Thus, our initial aim was to determine how real-time PCR can be used for the diagnosis and management of AK.

Earlier studies with multivariate analysis of AK classified AK into 5 stages according to the corneal depth of the lesion and the severity of the corneal involvement as as-