

14. Mauel MJ, Giovannoni SJ, Fryer JL: 1996, Development of polymerase chain reaction assays for detection, identification, and differentiation of *Piscirickettsia salmonis*. *Dis Aquat Org* 26:189–195.
15. Mauel MJ, Miller DL, Frazier K, et al.: 2003, Characterization of a piscirickettsiosis-like disease in Hawaiian tilapia. *Dis Aquat Org* 53:249–55.
16. Rodger HD, Drinan EM: 1993, Observation of a rickettsia-like organism in Atlantic salmon, *Salmo salar* L., in Ireland. *J Fish Dis* 16:361–369.

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Seasonal meningoencephalitis in Holstein cattle caused by *Naegleria fowleri*

Barbara M. Daft, Govinda S. Visvesvara, Deryck H. Read, Hailu Kinde, Francisco A. Uzal, Michael D. Manzer

Abstract. Primary amoebic meningoencephalitis is a fulminant infection of the human central nervous system caused by *Naegleria fowleri*, a free-living amoeba that thrives in artificially or naturally heated water. The infection usually is acquired while bathing or swimming in such waters. The portal of entry is the olfactory neuroepithelium. This report describes fatal meningoencephalitis caused by *N. fowleri* in Holstein cattle that consumed untreated surface water in an area of California where summer temperatures at times exceed 42°C. In the summers of 1998 and 1999, severe multifocal necrosuppurative hemorrhagic meningoencephalitis was observed in brain samples from nine 10–20-month-old heifers with clinical histories of acute central nervous system disease. Olfactory lobes and cerebella were most severely affected. Lesions were also evident in periventricular and submeningeal neuropil as well as olfactory nerves. *Naegleria fowleri* was demonstrated by immunohistochemistry in brain and olfactory nerve lesions and was isolated from one brain. Even though cultures of drinking water did not yield *N. fowleri*, drinking water was the likely source of the amoeba. The disease in cattle closely resembles primary amoebic meningoencephalitis in humans. *Naegleria* meningoencephalitis should be included among differential diagnoses of central nervous system disease in cattle during the summer season in areas with high ambient temperatures.

Key words: Bovine; encephalitis; *Naegleria fowleri*; primary amoebic meningoencephalitis.

Infection of the human central nervous system (CNS) with free-living amoebae was first described in 1966 and is now recognized worldwide.¹⁴ There are 4 genera of amoebae that cause CNS disease in mammals, namely *Acanthamoeba* (several species), *Naegleria fowleri*, *Balamuthia mandrillaris*, and the recently described *Sappinia diploidea*.¹² *Acanthamoeba* and *Naegleria* are ubiquitous in soil and fresh water, including lakes, streams, and hot springs. These amoebae have also been isolated from various artificial water sources, such as swimming pools, tap water, heating and ventilation units, air conditioners, cooling water, sewage, contaminated cell cultures, and contact lens-storing fluid.^{5,15,18} Their cysts have even been demonstrated in dust during dust storms.¹³ The habitat of *Balamuthia* is not known but is believed to be similar to that of *Naegleria* and *Acanthamoeba*.⁸

Primary amoebic meningoencephalitis (PAM) is the term

for the human disease caused by *N. fowleri*. It is an acute, usually fatal, necrotizing, and hemorrhagic meningoencephalitis.² As of 2001, 200 cases of PAM have been reported in humans. About half of these were reported in the United States (G. S. Visvesvara, personal communication). Although CNS infections due to *Acanthamoeba* and *Balamuthia* have been recorded in animals, such as dogs, sheep, cattle, primates, and horses,^{1,4,9–11,15,16} there has been only 1 report of naturally acquired PAM in animals, namely in a South American tapir at a zoo in Arizona.⁶ Primary amoebic meningoencephalitis has been experimentally induced in mice, sheep, and monkeys.^{12,17,18}

Naegleria fowleri is thermophilic and tolerates temperatures of up to 45°C; hence, the frequent association of PAM with a history of contact with naturally warm or artificially heated waters. Most human cases occur in healthy young individuals after swimming or bathing in pools, ponds, hot springs, canals, or lakes, although some cases have been associated with tap water or inhalation of cysts during dust storms.¹² The portal of entry is the olfactory mucosa. The parasite migrates to the brain via olfactory nerves. Incubation period is short, with onset of clinical signs several days following exposure. The disease rapidly progresses and usually culminates in death within 5–7 days.⁷

The life cycle of *N. fowleri* includes a trophozoite stage (amoebic form), a temporary flagellate stage, and, in unfavourable conditions, a cyst stage.

From the University of California, Davis, School of Veterinary Medicine, California Animal Health and Food Safety Laboratory System, San Bernardino Branch, San Bernardino, CA, 92408 (Daft, Read, Kinde, Uzal); Davis Central Reference Laboratory, Davis, CA, 95616 (Manzer); and Centers for Disease Control and Prevention, Division of Parasitic Diseases, Chamblee, GA, 30341 (Visvesvara).

Corresponding Author: Barbara M. Daft, California Animal Health and Food Safety Laboratory, San Bernardino Branch, 105 W. Central Avenue, San Bernardino, CA 92408.

avorable environments, a cyst stage. The cyst stage is susceptible to desiccation.² Only the trophozoite stage of *N. fowleri* has been detected in CNS lesions. This is in contrast with other free-living opportunistic amoebae, where identification of cyst stages aids in their differentiation from *Naegleria*.¹¹ The purpose of this report is to describe the clinical disease and lesions of PAM in cattle and to highlight environmental and management factors that presumably led to infection with this amoeba.

Between July 30 and September 28, 1998 and 1999, specimens from nine 10–20-month-old Holstein heifers with a history of acute CNS disease were submitted to the California Animal Health and Food Safety Laboratory System, San Bernardino Branch (CAHFS-SB). All submissions were from 1 large heifer-raising operation (approximately 10,000 animals kept on 3 ranches separated by several kilometers) located in southern California in an arid but highly productive agricultural area irrigated by Colorado River water supplied through a system of canals and ditches. The area is 25–31 m below sea level, has an average of 77 mm of precipitation per year and high ambient temperatures during the summer. The 3 ranches relied on untreated canal water for livestock drinking water. On 2 ranches, water flowed from a concrete canal into an earthen-walled holding pond, and from there, in underground pipes to concrete troughs approximately 60 cm deep. On the third ranch, drinking water flowed directly from a canal to troughs via underground pipes. There was a continuous flow of water through troughs on 2 ranches, while on the third, water level was controlled by a float. Cattle were also exposed to canal water by water sprayed from a tank truck to keep dust down in aisles, and canal water was used to reduce the dustiness of mixed feed.

Major feed ingredients were mixed on the ranch and consisted of locally produced irrigated forage and seed screenings. Other ingredients were distillery by-products, molasses, chicken manure, and mineral mix. Animals were kept in dry lots and were provided with shade.

Initial clinical signs were anorexia, depression, mucus-containing feces, and sometimes pyrexia and nasal discharge. After treatment with antimicrobials and/or other medications for 2–3 days, heifers appeared to improve but then deteriorated on day 4 or 5, showing various combinations of facial paralysis, circling, hypermetria, ataxia, weakness, head tilt, falling, blindness, seizures, and lateral recumbency. Of a total of twenty-one 5–20-month-old heifers with similar clinical signs in 1998, specimens from 6 animals were submitted to the laboratory. Three submissions from a smaller outbreak on the same premises were received in 1999.

Submitted specimens were as follows: entire fresh heads ($n = 7$), fresh and formalin-fixed brain ($n = 1$), live animal ($n = 1$). In a few cases, fresh or formalin-fixed lung, liver, kidney, colon, heart, rumen, abomasum, skeletal muscle, and small intestines were also submitted. Specimens were generally shipped to the laboratory on ice by courier overnight. One head was hand carried unpreserved to the laboratory in 1999.

Laboratory procedures included culture of brain as follows: aerobic cultures ($n = 9$), *Mycoplasma* ($n = 6$) and *Listeria* cultures ($n = 4$), anaerobic culture ($n = 3$) and fun-

gal culture ($n = 2$). Rabies was ruled out by fluorescent antibody testing of 4 brains. For histopathology, tissues were routinely processed and stained by hematoxylin and eosin or other stains (periodic acid–Schiff; Giemsa stain; Brown–Brenn-modified Gram stain; Steiner silver stain). Sections of ethmoid area from 2 animals were decalcified^a and processed as other tissues. The following CNS sections were examined: medulla ($n = 8$), cerebellum and olfactory bulb ($n = 7$), brain stem ($n = 6$), cerebrum ($n = 5$). Other tissues examined microscopically were lung and heart ($n = 4$); kidney and small intestines ($n = 3$); colon, retropharyngeal lymph node, and spleen ($n = 2$); abomasum, rumen, and skeletal muscle ($n = 1$).

Immunohistochemistry (IHC) for *Acanthamoeba*, *Balamuthia*, and *Naegleria* was performed as follows. Four-micron-thick sections of brain and ethmoid tissue were treated with pepsin and horse serum for antigen retrieval and blocking, respectively. This was followed with polyclonal anti-*Balamuthia*, *Acanthamoeba*, and *Naegleria fowleri* rabbit serum^{4,15} diluted 1:400, 1:800, and 1:1,000, respectively. After primary antibody, the avidin–biotin–peroxidase technique^b was used followed by a chromogen^c to produce red-brown positive staining of amoebae. Sections known to contain the above amoebae served as positive controls when available. Negative controls consisted of identical sections using diluted normal rabbit serum as primary antibody. Immunofluorescent (IF) procedure for *N. fowleri* was carried out by routine methods utilizing a fluorescent, conjugated polyclonal rabbit antiserum.⁶

In 1998, 5 brain specimens were submitted to the Centers for Disease Control (CDC) and consisted of fresh or frozen (-20°C) brain or brain in viral transport media containing amphotericin B and frozen at -70°C . In 1999, 2 fresh samples of brain suspended in Page amoeba saline¹⁴ and 1 sample of spinal fluid were cultured at CAHFS-SB and also sent to CDC for culture.

Water samples (4–8 liters) were collected on October 7, 1998, and August 10, 1999, at the 3 ranches. These samples were canal water from inlets to ranches, pond water, and water from troughs. In the laboratory, water was filtered through cheesecloth to remove coarse debris and centrifuged at $1500 \times g$ for 10 minutes. Aliquots of sediment were shipped to the CDC for culture (1998 and 1999) and parallel cultures were set up at CAHFS-SB (1999 only).

Cultures for *N. fowleri* at the CDC and CAHFS-SB were incubated at 37 and 42°C according to a method utilizing nonnutrient agar and *Escherichia coli* feeder culture.¹⁴ Direct wet mounts of cultures were examined for amoebae, and, if present, screened by IF for *N. fowleri*. CAHFS-SB cultures suspected to contain *Naegleria* were sent to the CDC for confirmation by immunofluorescence.⁶

Water temperature was recorded daily at 3 PM at 2 sites (a pond and a water trough) on 1 ranch from August 27 to October 10, 1999. Daily high ambient temperatures for 1998 and 1999 were obtained from the Western Regional Climate Center.

Gross brain lesions varied among animals. Some had multifocal meningeal hemorrhages and tan-gray thickening of meninges, most pronounced over ventral or ventrolateral brain stems and lateral cerebral surfaces. There often were

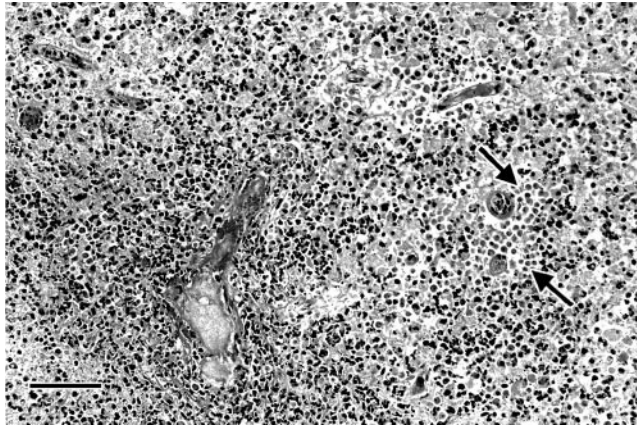


Figure 1. Bovine brain (olfactory bulb) with *Naegleria* meningoencephalitis. There is necrosis, thrombosis, and intense infiltrate of neutrophils. Note perivascular aggregates of amoebae (arrows). Hematoxylin and eosin. Bar = 100 μ m.

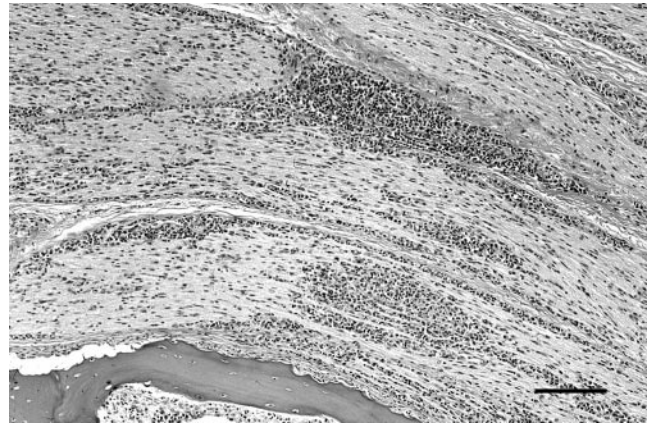


Figure 2. Bovine olfactory nerve from heifer with *Naegleria* meningoencephalitis. Note multifocal nonsuppurative olfactory neuritis affecting epineurium and endoneurium. Hematoxylin and eosin. Bar = 200 μ m.

tan-gray deposits obscuring cerebellar cortical surface. In 7 animals, brain parenchyma adjacent to the cribriform plate was unilaterally light brown and malacic. In addition, multifocal gray-tan discolorations were noted on sectioned surfaces of fixed cerebella, dorsal brain stems, hypothalamus, and periventricular areas. In cross sections at the rostral colliculi, lesions were sometimes clearly bilateral and nearly symmetrical. Middle and internal ears and cross sections of ethmoid tissue from 3 heifers had no gross lesions. There were no gross lesions in 1 animal's poorly preserved brain.

Histopathology revealed multifocal, necrosuppurative, and hemorrhagic meningoencephalitis. Most severely affected areas were the anterior cerebra, olfactory bulbs, and cerebella. In olfactory bulbs, lesions were unilateral in 6 animals and bilateral in 1 (this area was not examined in all animals). Affected olfactory bulb and adjacent brain had large areas of malacia that contained remnants of thrombosed vessels, proteinaceous fluid and fibrin, large numbers of neutrophils (many degenerate), rare multinucleated giant cells, and aggregates of amoebic trophozoites, frequently in perivascular areas (Fig. 1). Malacic areas were surrounded by zones of basophilic necrotic debris containing degenerate neutrophils and necrotic, thrombosed vessels suggestive of infarction. Ependyma and lateral ventricular space were multifocally obscured by necrosis and exudation. There was perivascular cuffing by lymphocytes and macrophages further away from malacic areas. Lesions in the cerebella were similar to those in olfactory bulbs, with involvement of entire folia and loss of the sharp demarcation between meninges and neuropil. Overlying meninges were thickened by infiltrates of lymphocytes, fewer macrophages, small numbers of neutrophils, fibrin, and red cells. In the medulla and midbrain, submeningeal and periventricular neuropil had lymphocytic perivascular cuffing, multifocal to coalescing infiltrates of epithelioid macrophages, occasional microabscesses, and intense meningitis. Ethmoid areas from 2 animals a few millimeters rostral to the cribriform plate revealed unilateral multifocal infiltrates of mononuclear leukocytes within nerve fascicles and epineurium (Fig. 2). Very slight similar inflammation

was noted in olfactory mucosa. Unaffected contralateral olfactory mucosa and nerve had no such lesions.

Amoebae were most easily recognized when clustered in aggregates or in perivascular spaces (Fig. 3). Amoebae were round to ovoid, surrounded by a clear space, 5–11 μ m in diameter, and had eosinophilic, slightly granular and vacuolated cytoplasm. The nucleus was small, poorly delineated, weakly basophilic, and often eccentrically located and had a prominent 1–3- μ m karyosome occupying about one third of the nuclear area (Fig. 3). None of the special stains were helpful in identifying amoebae. No significant histopathologic lesions were seen in heart, lung, kidney, liver, spleen, rumen, abomasums, colon, skeletal muscle, retropharyngeal lymph node, or small intestine.

Immunohistochemistry (IHC) or IF for *N. fowleri* was positive in all 9 animals (Fig. 4). *Naegleria fowleri* was demonstrated in small numbers by IHC in olfactory nerves from 2 animals. *Naegleria fowleri* was isolated only from the hand-carried brain, cultures of which were started at

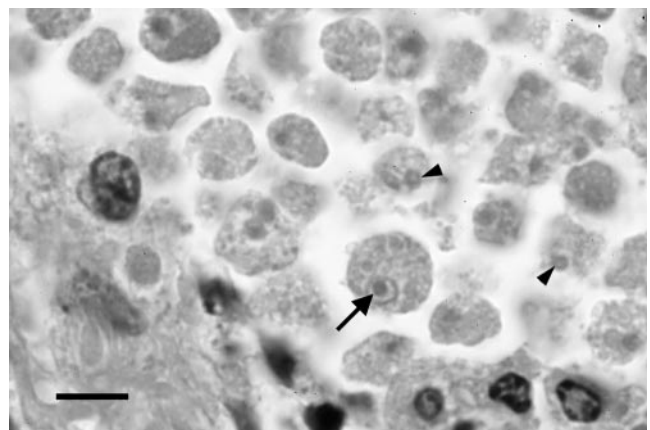


Figure 3. Bovine brain (olfactory bulb) with *Naegleria* meningoencephalitis. A cluster of amoebae are recognizable by their karyosomes (arrowheads). Nucleus and karyosome are visible in 1 amoeba (arrow). Hematoxylin and eosin. Bar = 10 μ m.

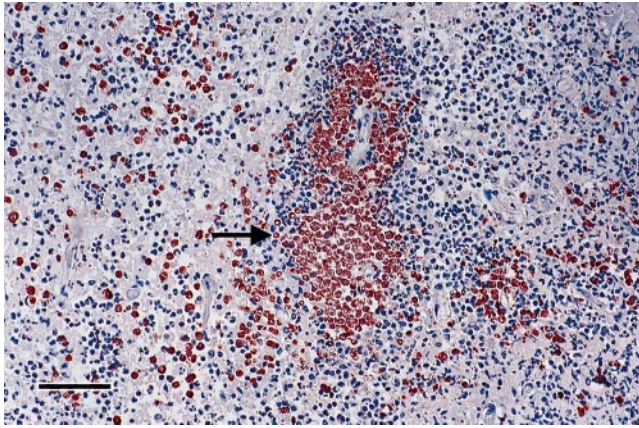


Figure 4. Immunohistochemical stain of bovine brain (olfactory bulb) with *Naegleria* meningoencephalitis. Note numerous brown-staining *N. fowleri* around vessels (arrow) and within neuropil. Bar = 100 μ m.

CAHFS-SB within a few hours of arrival. All other cultures of brain and water specimens were negative for *N. fowleri*, although some specimens of water yielded other types of amoebae, including other *Naegleria* spp. All bacterial and fungal cultures of brain were negative.

Pond and trough water temperatures taken at 3 PM on 1 ranch reached 32.2°C for 4 days at the end of August and again 32.2°C during mid-September, 1999. Highest recorded temperatures were 33.1°C in a water trough and 35°C in pond water. Monthly average daily high ambient temperatures were 42, 42.2, and 39°C for July, August, and September, 1998, respectively. For 1999, temperatures were 40, 41, and 39.8°C for the same months.

This is the first documented case of PAM in livestock. The other natural case of PAM in animals was diagnosed in Phoenix, Arizona, an area with similar high ambient summer temperatures. Even though isolation attempts for *N. fowleri* from drinking water were unsuccessful, the source of infection for these heifers was probably the drinking water. The disease is seasonal, associated with high ambient temperatures and elevated water temperatures, which favor proliferation of *N. fowleri*. Water temperatures in water troughs reached 32°C, a temperature slightly higher than that reported for outbreaks in humans bathing in water with average summer water temperatures of 28°C (Florida) and 31°C (Australia).³ Heifers licking their nostrils may have transferred amoebae into the nasal cavity. It is also known that *Naegleria* migrates in nasal mucus. Interview of workers at the ranches did not suggest that cattle were immersing nostrils in drinking water or otherwise playing with water on hot days. In humans, immersion of nostrils in water may not be necessary for infection, as some cases occurred while washing faces without immersion.² Other possible sources of infection may have been water sprayed for dust control. If cattle were feeding while aisles were sprayed, deep inhalation of spray is possible. However, the literature does not mention human cases associated with sprayed water. Feed as a source of inhaled *Naegleria* was unlikely because the disease is seasonal and similar feed and feed sources are used all year. Locally produced feed is shipped to distant ranches

and farms that use well or city drinking water; however, the disease has not occurred on these premises to our knowledge, even though other management factors and ambient summer temperatures may be similar. In humans, infection due to dust-borne *Naegleria* is rarely reported.

Human cases of PAM have been reported in the same general area. In August and September of 1990, 5 cases of PAM were reported in Mexicali, Mexico, approximately 35 miles south of the premises in this report. Affected people had bathed in a canal transporting Colorado River water, from which *N. fowleri* was subsequently cultured.⁵

There are several reasons for the failure to isolate the pathogen in all but 1 brain sample. In 1998, water was collected in October when temperatures were lower than during the summer. Brains were shipped to the CDC in viral transport media containing amphotericin B, to which *N. fowleri* is very sensitive and which is used for treatment of the infection in humans.² Some of the brains were frozen at -20°C or shipped with ice, both of which are temperatures not favorable to survival of *Naegleria* (G. S. Visvesvara, personal communication). Failure to isolate *N. fowleri* from water samples in 1999 is unexplained.

Lesions of PAM in cattle resemble very much those described in humans.² In cattle, the likely portal of entry was also the nasal mucosa and olfactory nerves, as evidenced by severe olfactory bulb and adjacent cerebral lesions, multifocal nonsuppurative rhinitis with olfactory nerve neuritis, and demonstration of *N. fowleri* within the olfactory nerve. In humans, the parasite is thought to reach the brain via the mesaxonal space of the olfactory nerve. In the brain, amoebae then disseminate via the subarachnoid space and ventricles.⁷ In the bovine disease, lesions in some areas tended to be superficial, bilateral, and symmetrical, also suggesting dissemination of the parasite in subarachnoid space, which is known to communicate with the perivascular space of vessels entering the brain parenchyma.

The diagnosis of *Naegleria* meningoencephalitis is difficult. In the bovine, common differential diagnoses include cerebral abscesses, bacterial meningitis, thrombotic meningoencephalitis (TME), and polioencephalomalacia. Grossly, TME most closely resembles *Naegleria* meningoencephalitis. The 2 diseases can be differentiated by grossly visible discoloration of the olfactory area in *Naegleria* meningoencephalitis. However, the olfactory portion of the brain may be inadvertently destroyed or not removed from the cranium, and for this reason, olfactory lesions, though present, may not be observed. *Acanthamoeba* may also enter the brain via the olfactory nerve but does so rarely.⁷ It usually enters the brain via the blood stream.

Microscopically, amoebae are difficult to identify because they resemble degenerate macrophages and may be few in number. The tendency of the amoebae to accumulate in aggregates or in perivascular spaces within areas of necrosis is most helpful, as is the small, weakly basophilic karyosome within a poorly delineated, small nucleus. There are very few or no multinucleated giant cells or eosinophils to direct the pathologist toward parasitic etiology. Some amoebae form cysts, visualized by periodic acid-Schiff stain, but *N. fowleri* does not, possibly because patients' death occurs during the acute stage. Stains to detect bacteria are also not

useful. Finally, it is advantageous to know that the disease is seasonal, occurs in areas with high ambient summer temperatures, and may be associated with the use or consumption of untreated surface water.

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Sources and manufacturers

- a. CalEx II, Fisher Scientific, Pittsburg, PA.
- b. Vector Laboratories, Inc., Burlingame, CA.
- c. 3-amino-9-ethylcarbazole, Dako Corporation, Carpinteria, CA.

References

1. Fuentealba IC, Wikse SE, Read WK, et al.: 1992, Amebic meningoencephalitis in a sheep. *JAVMA* 200:363–365.
2. John DT: 1982, Primary amebic meningoencephalitis and the biology of *Naegleria fowleri*. *Ann Rev Microbiol* 36:101–123.
3. De Jonckheere JF: 1975, The effect of thermal pollution on the distribution of *Naegleria fowleri*. *J Hyg Camb* 75:7–13.
4. Kinde H, Visvesvara GS, Barr BC, et al.: 1998, Amebic meningoencephalitis caused by *Balamuthia mandrillaris* (leptomyxid amoeba) in a horse. *JVDI* 10:378–381.
5. Lares-Villa F, De Jonckheere JF, De Moura H, et al.: 1993, Five cases of primary amebic meningoencephalitis in Mexicali, Mexico: study of the isolates. *J Clin Microbiol* 31:685–688.
6. Lozano-Alarcon F, Bradley GA, Houser BS, Visvesvara GS: 1997, Primary amebic meningoencephalitis due to *Naegleria fowleri* in a South American tapir. *Vet Pathol* 34:239–244.
7. Martinez AJ, Visvesvara GS: 1997, Free-living, amphizoic and opportunistic amebas. *Brain Pathol* 7:583–598.
8. Martinez AJ, Visvesvara GS: 2001, *Balamuthia mandrillaris* infection. *J Med Microbiol* 50:205–207.
9. McConnell EE, Garner FM, Kirk JH: 1968, Hartmannellosis in a bull. *Path Vet* 5:1–6.
10. Pearce JR, Powell HS, Chandler FW, Visvesvara GS: 1985, Amebic meningoencephalitis caused by *Acanthamoeba castellanii* in a dog. *JAVMA* 187:951–952.
11. Rideout BA, Gardner CH, Stalis IH, et al.: 1997, Fatal infections with *Balamuthia mandrillaris* (a free-living amoeba) in gorillas and other Old World primates. *Vet Path* 34:15–22.
12. Schuster FL, Visvesvara GS: 2004, Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *Intern J Parasitol* 34:1001–1027.
13. Szenasi Z, Endo T, Yagita K, Nagy E: 1998, Isolation, identification and increasing importance of free-living amoebae causing human diseases. *J Med Microbiol* 47:5–16.
14. Visvesvara GS: 1999, Pathogenic and opportunistic free-living amoebae. In: *Manual of clinical microbiology*, ed. Murray PR, Baron EJ, Pfaller MA, et al., 7th ed., pp. 1383–1390. ASM Press, Washington, DC.
15. Visvesvara GS, Martinez AJ, Schuster FL, et al.: 1990, Leptomyxid ameba, a new agent of amebic meningoencephalitis in humans and animals. *J Clin Microbiol* 28:2750–2756.
16. Visvesvara GS, Schuster FL, Julio-Martinez A: 1993, *Balamuthia mandrillaris*, N. G., N. Sp., agent of amebic meningoencephalitis in humans and other animals. *J Euk Microbiol* 40:504–514.
17. Wong MM, Karr SL, Balamuth WB: 1975, Experimental infection with pathogenic free-living amoebae in laboratory primate hosts. I (A) A study on susceptibility to *Naegleria fowleri*. *J Parasitol* 61:199–208.
18. Young MD, Willaert E, Neal FC, Simpson CF, et al.: 1980, Experimental infection of sheep with *Naegleria fowleri* of human origin. *Am J Trop Med Hyg* 29:476–477.