

Granulomatous Meningoencephalitis *Balamuthia mandrillaris* in Peru: Infection of the Skin and Central Nervous System

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ABSTRACT

Balamuthia mandrillaris is an emerging cause of sub acute granulomatous amebic encephalitis (**GAE**) or *Balamuthia mandrillaris* amoebic infection (**BMAI**). It is an emerging pathogen causing skin lesions as well as CNS involvement with a fatal outcome if untreated. The infection has been described more commonly in immunocompetent individuals, mostly males, many children. All continents have reported the disease, although a majority of cases are seen in North and South America, especially Peru.

Balamuthia mandrillaris is a free living amoeba that can be isolated from soil. In published reported cases from North America, most patients will debut with neurological symptoms, where as in countries like Peru, a skin lesion will precede neurological symptoms. The classical cutaneous lesion is a plaque, mostly located on face, knee or other body parts. Diagnosis requires a specialized laboratory and clinical experience. This Amoebic encephalitis may be erroneously interpreted as a cerebral neoplasm, causing delay in the management of the infection. The diagnosis of this infection has proven to be difficult and is usually made post-mortem but in Peru many cases were pre-mortem. Despite case fatality rates as high as > 98%, some experimental therapies have shown some success with the use of azoles, flu cytosine, pentamidine, sulfadiazine, and synergistic anti-protozoal therapy with macrolides and phenothiazines. We have had experience with the use of Miltefosine and it has been successful.

BALAMUTHIA MANDRILLARIS

History

Balamuthia mandrillaris was isolated in the spring of 1986 from an ulcer in the cerebrum of a 3-year-10 month old pregnant mandrill baboon (*Papio sphinx*) that died of a neurological disease at the San Diego Zoo Wild Animal Park. *B. mandrillaris* was initially described as a leptomyxidameba [1].

It was initially identified as a leptomyxid (*Leptomyxa* and *Gephyramoeba*) amoeba because it resembles, especially in the cyst stage, many features that are typical of the amoebae included in the familyleptomyxidae. But, in the year 1993, the Dr G Visvesvarae et al., it was recognized as being different from the leptomyxids and was designated as a new genus and species, *Balamuthia mandrillaris* [1,2]. It is the only species included in the genus *Balamuthia*.

The *Balamuthia mandrillaris* is named in honor of the late professor William Balamuth (1914-1981) of the University of California at Berkeley, an eminent protozoologist known by his contributions to the studies in protist and free-living amoebas [3]. The Infection caused by this amoeba is similar to *Acanthamoeba*. *Balamuthia* causes an insidious, chronic, and subacute BMAI in both immunocompromised and immunocompetent individuals that may last anywhere from several weeks to 2 years [4-6].

In the Year 2000, Amaral Zettler et al., showed that *Balamuthia* does not belong to the order Leptomyxida and transferred *B. mandrillaris* to the Acanthamoebidae based on molecular biological studies [7].

Booton et al., 2003 [8] showed, using molecular biologically comparison of 7 *B. mandrillaris* sequences with 11 *Acanthamoeba* sequences, that *B. mandrillaris* is a sister genus to *Acanthamoeba*. They found no evidence for the possibility, that *B. mandrillaris* could be a new species of *Acanthamoeba* so the hypothesis that *Balamuthia* is an independent genus was assured. Nonetheless, the genus *Balamuthia* is a close relative of the monophyletic genus *Acanthamoeba*.

Therefore, according to the new taxonomic schema therefore *Balamuthia* is classified with *Acanthamoeba*, and is placed in the family Acanthamoebidae, Amoebozoa [9].

BIOLOGY

Life Cycle

Balamuthia mandrillaris - the life cycle is summarized in the graphic (Figure N° 1).

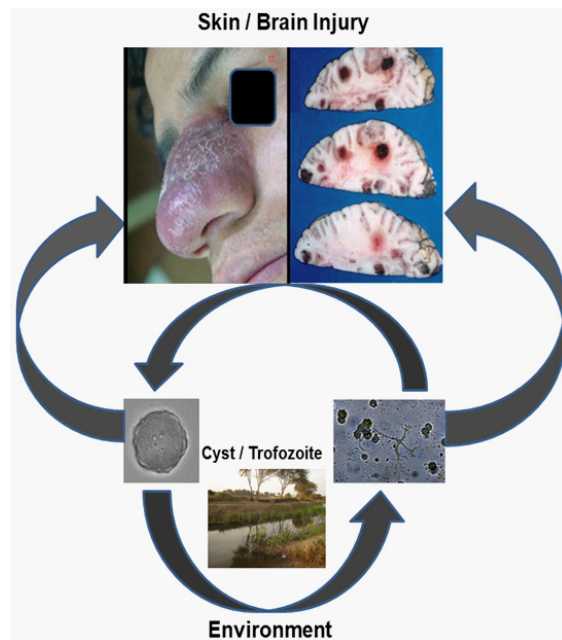


Figure N°1: The life cycle of *Balamuthia mandrillaris*. Trophozoite and Cyst in the environment.

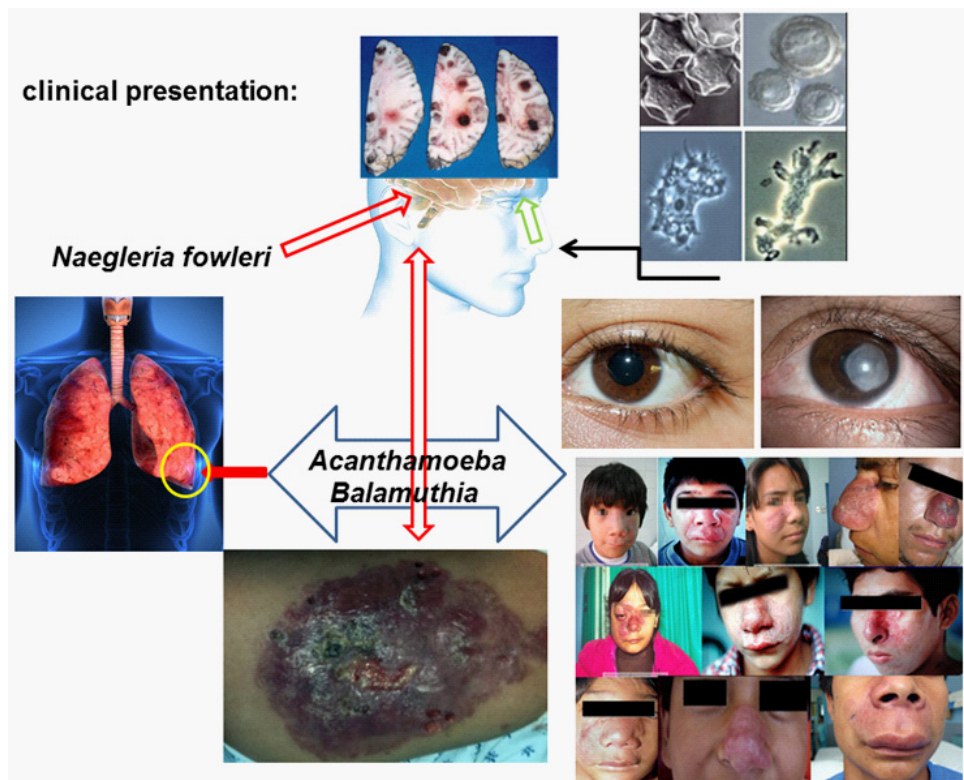


Figure N°2: Clinical presentations infections free-living amoebae.

B. mandrillaris is a free-living amoebae with a life cycle of two stages: cyst and trophozoite. Encystation allows the amoeba to resist environmental stresses, mostly lack of food, desiccation or changes in the environment that compromise the very existence of the trophozoite. Cyst diameter ranges from 12 to 30 microns; the endocyst is rounded and covered by the ectocyst, which can be very thick making the cyst seen as double walled under light microscopy. It is trilaminar, as revealed by electron microscopy [4]. Trophozoite stage is the infective form and its dimensions range from 12 to 60 microns, flattened and rounded with many spiny subpseudopodia giving them the general appearance of the Acanthamoebidae family. The *Balamuthia* pseudopodia varies in number but there are no more than 10 and they move independently, change in number, thickness and length, contain multiple nuclei and are used for feeding. *B. mandrillaris* may switch its movement from crawling to crab like “walking” resulting in a speed of 0.15 mm/s in monkey kidney cells culture [4,10]. *Balamuthia mandrillaris* feeds on other amoebas, not bacteria. By comparison, they grow slowly, taking weeks before the amoebas are recognized microscopically amongst other amoebas, ciliates, worms and fungi. The use of the small amoeba to serve as an intermediate food source helps to reduce an overwhelming load of bacteria prior to the growth of *Balamuthia in vitro* [10]. The environmental source of *B. mandrillaris* is not well understood, since worldwide it has been isolated only sometimes.

MORPHOLOGY AND TAXONOMY

B. mandrillaris trophozoites were extremely pleomorphic, most of them exhibited an either rounded or elongated shape. The trophozoite are pleomorphic and measures 12–60 µm with a mean of about 30 µm. But recently in Iran, [11] found *Balamuthia* trophozoites that were approximately 30–120 µm in diameter with dendritic pseudopodia and most of the time their trophozoites appeared stretched out and branched.

The trophozoites usually are uni-nucleated, but binucleate forms occasionally are seen. The nucleus contains a large, centrally placed, dense nucleolus, and occasionally trophozoites with two or three nucleolar bodies are seen, especially in infected tissues (Figure 2). Cysts are also uninucleated, more or less spherical, and range in size from 12 to 30 µm, with a mean of 15 µm. *Balamuthia* exhibits two types of locomotion; in one type it moves by typical amoeboid locomotion in cultures. In the second type it exhibits a spider-like locomotion on extended leg-like pseudopodia when feeding on tissue culture [12]. It has a cyst stage but lacks a flagellate stage.

Under the microscope light, cysts appear to be double-walled, with a wavy outer wall and a round inner wall. Ultrastructurally, however, the cysts possess three walls: an outer thin irregular ectocyst, an inner thick endocyst, and a middle amorphous fibrillar mesocyst. However, the forms observed in the environment have not been seen directly. So what if you can see them? Are the forms taken in the monoxenic culture axenic and cell culture (Figure 3&4).

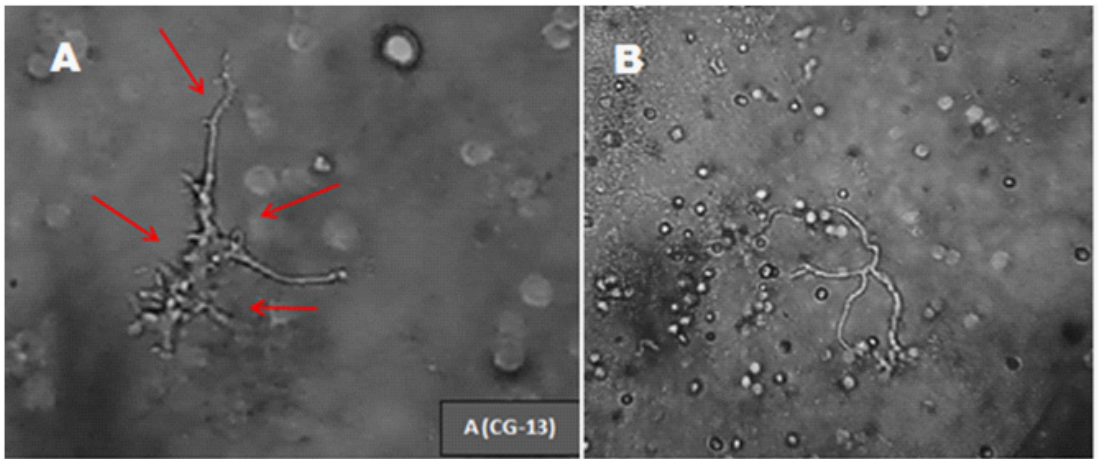


Figure N° 3: A) *Balamuthia*, trophozoite in monoxenic culture strain CG-13. Large polymorph trophozoite in monoxenic culture.

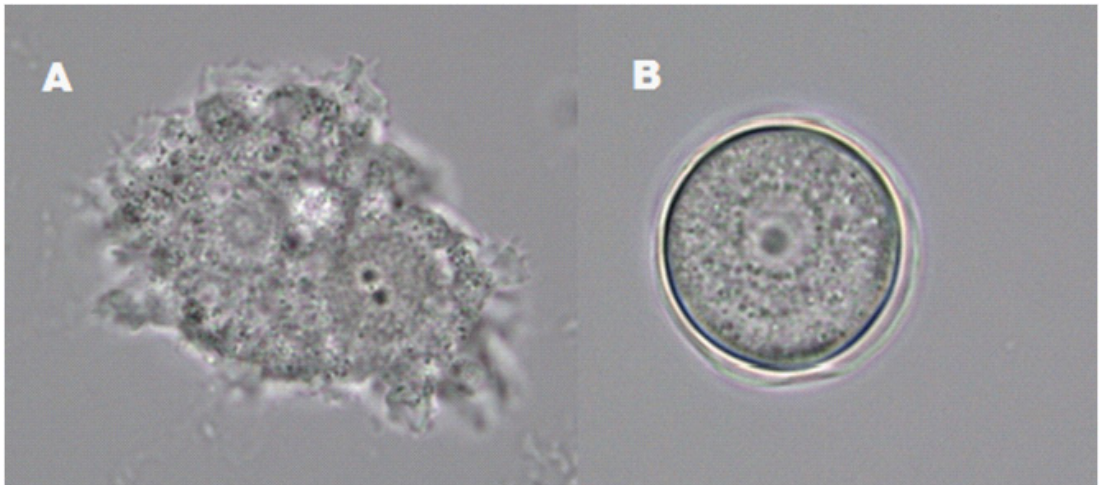


Figure N° 4: Axenic culture of *Balamuthia* isolate from sand of *Piura* (cost north from Peru). A) Trophozoite. B) Cyst. 100 X.

Table N°1: Revised classification of aerobic naked amoebae of phylum Amoebozoa. Two more clinical important amoeba. (From Smirnov AV, 2011).

Class Tubulinea Smirnov et al., 2005 em. (=Lobosea Cavalier-Smith, 2004)
Order Leptomyxida (Pussard and Pons, 1976) Page, 1987
Family Leptomyxidae (Pussard and Pons, 1976) Page, 1987. Leptomyxa, Rhizamoeba
Family Flabellulidae Bovee, 1970 em. Page, 1987. Flabellula, Paraflabellula
Family Gephyramoebidae Pussard and Pons, 1976. Gephyramoeba
Class Discosea Cavalier-Smith in Cavalier-Smith et al. (2004) em.
Subclass Longamoebia Smirnov and Cavalier-Smith subcl. n.
Order Centramoebida Rogerson and Patterson, 2002 em. Cavalier-Smith, 2004
Family Acanthamoebidae Sawyer and Griffin, 1975. <i>Acanthamoeba</i> , <i>Protacanthamoeba</i>
Family Balamuthiidae Cavalier-Smith in Cavalier-Smith et al., 2004. <i>Balamuthia</i>

CULTIVATION

Dunnebacke T et al., 2004 [10] was the first to isolate *Balamuthia* from soil samples in California. *Balamuthia mandrillaris* cannot be cultured on agar plates coated with bacteria. However, *Balamuthia* has been isolate from the environment 10 times [10,12-21], using agar plates nonnutritive. Recently, Tapia et al., 2013 [21]. It has been documented that *Balamuthia* is able to consume a wide variety of eukaryotes, but did not feed on *Acanthamoeba* cysts or algae, *Balamuthia* amoebae can be maintained *in vitro* on smaller free-living amoebae, the assumption is that they are a likely food source in nature, But the real food source of these organisms is not yet know. *Balamuthia* has been isolated from soil and dust samples worldwide. Infection is thought to be acquired by exposure of mucus membranes or non-intact skin to cysts or trophozoites from soil, as the strain from Peru (KJ439568- KJ439571) [22]. Update, *Balamuthia* has been recovered from that source water (KU184268, KU184269) [11]. The water may also be a mode of transmission. All of them isolated in culture grow at 37°C.

Culture cell Medium

B.mandrillaris growth on tissue culture cells. The amoebae have been cultured on monolayers of tissue culture cells. It possible to use different lines of tissue culture cells: *African green monkey kidney, rat glioma, mouse macrophage, murine mastocytoma, human lung fibroblasts and human brain microvascular endothelial cells*.

The technique for isolating it from the skin lesion, brain tissue or cerebrospinal fluid (CSF) is to add macerated brain tissue or CSF to the tissue culture [1,23]. After a long period from days to several weeks, amoebae will start feeding from the monolayer, initially producing a few plaques like those produced by a virus and soon consuming virtually all the tissue culture cells [1,4,12] (Figure N° 5).

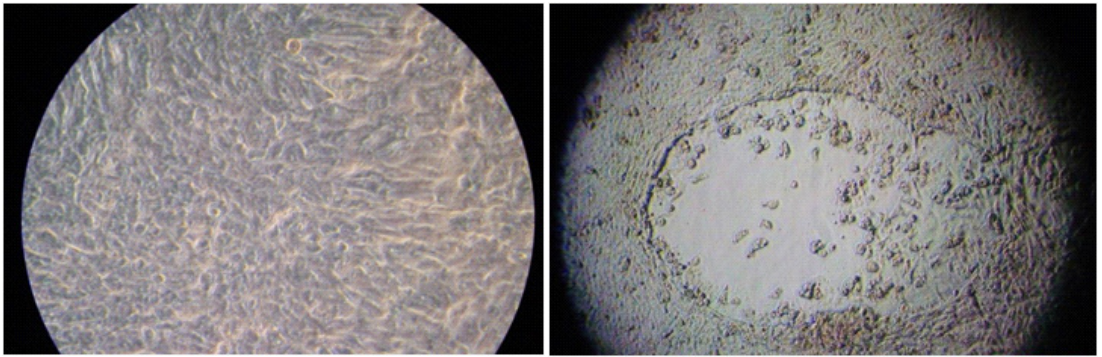


Figure N ° 5: The destruction of the monolayers by a strain of *Acanthamoeba* T-4 in culture cell of African green monkey kidney.

Axenic medium

There are very few culture media for the group of Gymnamoebae. *Acanthamoeba*, *Naegleria* and *Balamuthia* can grow well in cell lines, but not all strains can grow in liquid medium. The Cerva's medium, consisted in 10.0 g of Bacto™ Casitone (Difco, Detroit, Michigan, USA) in a final volume of 500 mL of ddH₂O sterilized by autoclave, supplemented with 10% (v/v) fetal bovine serum, 200 UI/mL penicillin, and 200 µg/mL streptomycin as antibiotics, was used as a basal medium [24]. This medium has served as the base medium for the development of culture media for other strains of amoebae such as *Balamuthia* [24].

In 1996, Schuster developed a new medium for axenic culture for *B.mandrillaris* growth in an enriched cell-free axenic medium (BM-3),supplemented with fetal calf serum, vitamins, nonessential amino acids, liver extract, yeast RNA, and hemin (see Table 1; [25]. The doubling time with BM-3 medium was 38.46 hours.

Until now, complex media such as the BM-3 culture medium and the modified Chang's special medium are the only ones that have been recommended for axenic cultivation of *B. mandrillaris* [25,26]. BM-3 medium is excellent for massive growth of *B. mandrillaris* due to its richness in nutrients.However, there is a recent medium re designed by the Mexican group, which reduces costs but takes more time in isolation [27].

Table N° 2: Formulation of a cell-free medium (BM-3) for axenic growth of *Balamuthia mandrillaris* (Schuster and Visvesvara, 1996).

Compounds (Medium BM-3)	Concentration
Base Medium	
Biosate peptone (BBL)	2,0 g
Yeast extract (Difco)	2,0 g
Torula yeast RNA (Sigma)	0,5 g
Glass-distilled water	345,0 ml
Supplements	
Hank's balanced sales 10X	34,0 ml
5% ox liver digest in Hanks' salts	100,0 ml
MEM vitamin mixture, 100x	5,0 ml
Lipid mixture, 1,000x (Sigma)	0,5 ml
MEM nonessential amino acids, 100X (Sigma)	5,0 ml
10% Glucose	5,0 ml
Hemin at 2 mg/ml (Mann Research)	0,5 ml
0.5% Taurine (Sigma)	5,0 ml
Newborn calf serum (Gibco) to give 10%	50,0 ml

Table N°3: The Mexican team has developed a more economical means of cultivation and with good results [27].

Compounds (Lares-Jiménez medium)	Concentration
Bactocasitone (Difco)	10g
Hank's balanced sales 10X (Gibco)	34 ml,
H ₂ O (d)	456 ml
Newborn calf serum	10%
Penicillin y	400 U/ml
Streptomycin	400 µg/ml

MICROBIAL ECOLOGY

Balamuthia mandrillaris was isolation from soil and water, it is clear and there is evidence that *Balamuthia* is a protozoaand is a free-living amoeba.

The isolation of *Balamuthia* from soil on nonnutrientagar plates is possible with bacteria, successive growth waves of ciliates, soil amoebae (*Acanthamoeba*, Naegleria, Hartmannella, etc.), fungi and nematodes preceded the appearance of *Balamuthia*. But it is true that the delay in growth was likely due to the prolonged generation times of *Balamuthia* 32-52 hrs (date from *in vitro*).

Balamuthia has been isolated from soil on several occasions, but rarely has succeeded in axenization. It is important to highlight in others studies, the isolation and identification of *B.*

mandrillaris. So far, this species has been isolated ten times: five times from soil sources [10,12-14,18,20,22,28] others from water [11,17,19,29] and from dust samples in Iran [13,20]. Only in one of these cases, which occurred in the USA [28]. The isolate was associated with the death of a child due to BMAI. The studies from Ahmad et al. (2011) showed that sixteen of 17 Californian soil samples were positive compared with 0/44 from the United Kingdom [14].

Recently, Cabello-Vílchez et al. (2014) reported the environmental isolation of four *B. mandrillaris* strains from soil sources in Peru, the second country worldwide in number of BMAI reported cases world wide. The soil samples were collected from areas where clinical cases of BMAI were reported previously and therefore soil could be a source of infection in the coast of Peru [22].

The presence of *B. mandrillaris* does not appear to be restricted to fertilized soil or specific soil types [10, 22], but we ignore the physicochemical characteristics of these soils. There is very little information about the characteristics and soil conditions in which *Balamuthia* usually appears. In Mexico seven strains of *Balamuthia mandrillaris* of water from Nainarilagoon were isolated [16]. In the case of Peru is land for agriculture and desert sand. In Iran soil recreation parks.

PROTOZOOLOGY AND GLOBAL WARMING

Goldfuss, in 1817, first used the word “protozoa,” which was given modern meaning in 1845 by Siebold. Protozoans are microscopic one-celled organisms that are categorized according to their method of movements; Amoeba –include the pathogenic amoeba *Entamoeba* and *Endolimax* which cause dysentery in humans. But also free-living amoebae including *Acanthamoeba*, *Balamuthia mandrillaris* and *Naegleria fowleri*.

The free living amoeba constitute a large group, inhabiting fresh, brackish and salt water, moist soil and decaying vegetation. Some are coprozoic. This large and diverse group is separated into two groups on the based of their ability to undergo transformation from an amoeba to a flagellate stage. *Naegleria sp.* belongs to the family Vahkampfiidea, members of which are characterized as amoebaflagellates able to assume a temporary flagellate form while completely devoid of flagella in their amoeboid stage. *Acanthamoeba* and *Balamuthia mandrillaris*, belonging to the family Acanthamoebidea, never produce flagella. Most cases of primary amebic meningoencephalitis can be ascribed to infection with Naegleria; *Acanthamoeba sp* and *Balamuthia mandrillaris* are involved in chronic meningoencephalitis.

The importance of amoebae in the soil environment relies ontheir energetic connection between the microorganisms and the soil micro-fauna [30] as they become an important step in the matter of recycling and energy transformation in the soilenvironment [31]. Since amoebae are so diverse in terms of the kind of organisms they can prey upon, then their overall classification as bacterivores is an oversimplification of the role they play in soils. This latter has an important implication for environmental and epidemiology studies on pathogenic free-livingamoebae, since it will be a mistake trying to isolate a pathogenicspecies by providing the wrong food source.

In other words,utilizing bacterized cultures limit the environmental search tobacterivorous and omnivorous amoebae, leaving undetected allother tropic groups amoebae. *N. fowleri* feed on bacteria (bacterivoreamoeba), preferring gram negative bacteria over gram positive [32].

Climate change will also affect the frequency and strength of the “El Niño” events. It normally takes place every 4 years affecting the west part of the American continent, from the coast of Peru and Chile in the south up to the coasts of California in the northern Hemisphere [33-35]. Air and Oceans warming up produce atmospheric instabilities and the change of the ocean’s surface currents. Then heavy rainsin the inlands and the increase of productivity in the shores are the consequence of these changes in South America. Heavy rains produce floods in the tropical forests as well as in the deserts, causing troubles for human communities. Flooded arid lands receive an extra input of organic matter allowing the increase of productivity while the soil water reservoir lasts. Thus, it is easy to understand that the high number of cases in the American continent is concentrated in Peru and California. The greater proportion of isolation of *Balamuthia* has been done in on the coasts of the American continent. [15].

MECHANISMS OF PATHOGENESIS

Mechanisms Described

The clinical manifestations of patients infected with either *Acanthamoeba* spp. or *B. mandrillaris* are similar and will be considered together. *Balamuthia* amebae can develop in both apparently healthy and immu-nosuppressed individuals (associated with HIV-related cases, alcoholism, drug users, and malnutrition cases [36,37]. Like *Acanthamoeba*, *Balamuthia* amebae probably spread through the bloodstream and cross the BBB to cause granulomatous encephalitis.

In vitro studies on the invasion of *B. mandrillaris* using human brain microvascular endothelial cells have shown that attachment of the parasites to endothelial cells is mediated by a galactose-binding protein on their surface [36, 38]. This binding might lead to release of amoeba proteases that degrade basement membranes and tight junction proteins to facilitate passage of the parasite across the BBB [38,39].

Exposition of previous inflamed nasal mucosa or skin lesionsmay be conditions needed for *B. mandrillaris* invasion [40,41]. Recently it has been shown that *Balamuthia* induces human brain micro vascular endothelial cells to release a pleiotropic cytokine, IL-6 [42], which is known to play a role in initiating early inflammatory response [38]. Further more, it has been shown that *B. mandrillaris* interacts with the host connective tissue containing collagen-1, fibronectin, and laminin-1, which is probably related to the production of food cups thus enhancing the pathological process [43].The proteases destroy the extracellular matrix (collagen, elastin and plasminogen) that hold brain cells together and facilitate penetration into the brain parenchyma. Additionally, other enzymes - lipase, phospholipase A and lysophospholipase A - may also be involved [44,45].

The incubation period is unknown, although, in disseminated infections, several weeks or months may elapse between the appearance of cutaneous lesions and the recognition of CNS disease. Neurologic symptoms develop insidiously, and most patients have focal deficits or altered mental status, sometimes with headache, meningismus, nausea, vomiting, lethargy, or low-grade fever, hemiparesis, visual disturbances, facial nerve palsy, ataxia, and other cerebellar signs. Over the course of a week to several months, the disease progresses to coma and eventually death from increased intracranial pressure and brain herniation or from secondary infection and multiorgan failure [46].

HYPOTHETICAL MECHANISMS

Balamuthiasis: Disease in Human and animals

B. mandrillaris have been isolated from skin and soil (Skin lesions are the rule in the cases from Peru) and brain biopsy material (in many countries around the world). The disease occurs with or without skin lesion. Khan and Lorenzo-Morales, they ask themselves questions that to this day are not easy to answer, the questions are: why are there more skin lesions south america reports than in the northern hemisphere?, Is *B.mandrillaris* ubiquitous?, Why Is the central face lesion only common in Peru? And Are there other species/genotypes of this genus?

Probably some questions could be partially answered. The usual locations of the lesion on the central face area that were reported from Peru are the same as those described in cases from Australia, Thailand and Chile. [47-49]. This is very interesting because they are countries that receive high doses of ultraviolet radiation and is the most exposed anatomical area of the face. (Figure N°6).

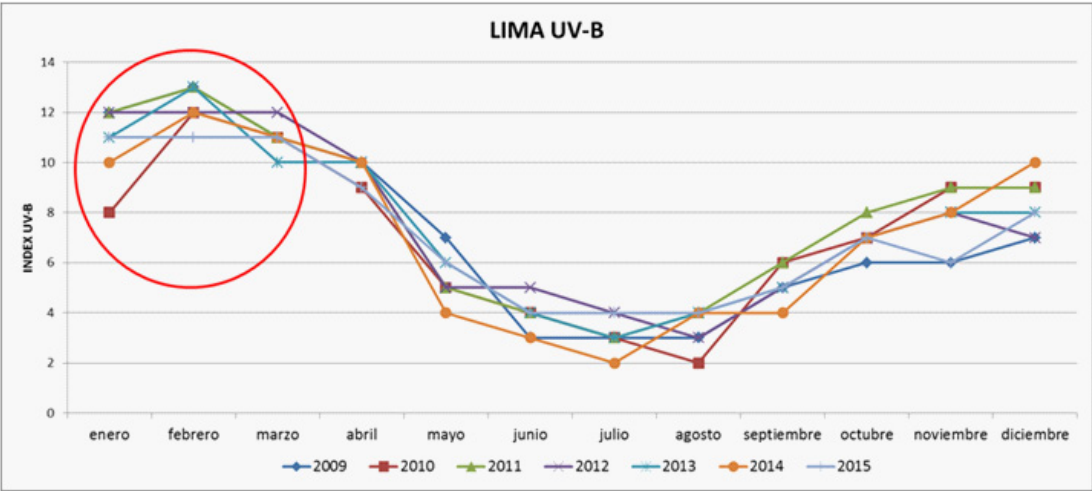


Figure N°6: Index of levels of Radiation in the capital of Peru. Summer is the period of highest intensity of UV-B radiation (Red circle). Data obtained from monthly bulletins issued by the SENAMHI.

B. mandrillaris has been isolated from soil of Peru in many opportunities. Therefore, it is probable that the soil allows the development of *Balamuthia mandrillaris* due to some factor unknown at the moment, but the El Niño–Southern Oscillation [ENSO] could be a predisposing factor due to the climate turbulence that suffers the American continent.

Central face lesions are a common factor in Peru, probably because the nasal cavity is colonized by many free-living amoebae including *B.mandrillaris* (Figure N° 9).

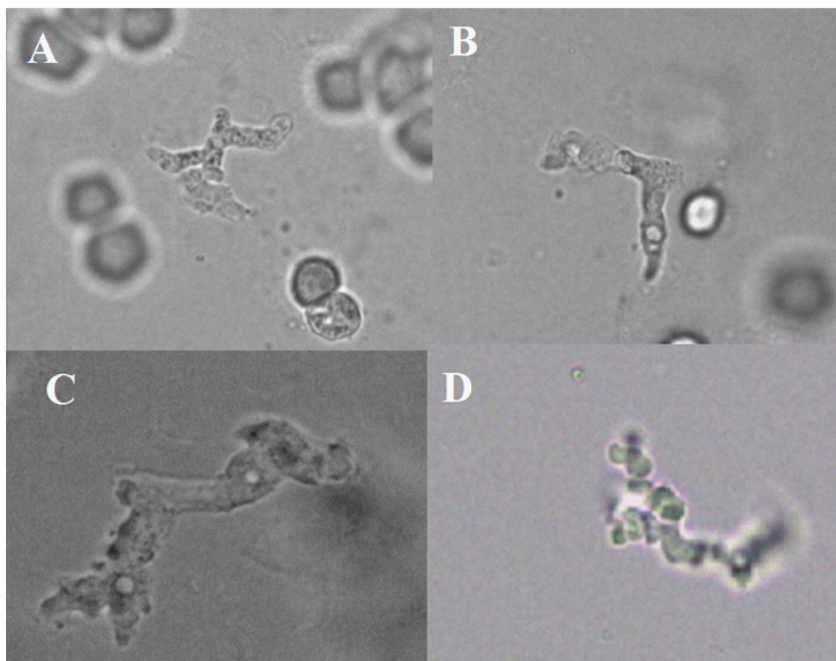


Figure N°7: *Balamuthia* –like the isolated from nasal mucosa from Peru. *Balamuthia* on non-nutritive agar plate (A, B, C, D, E) 6 weeks of culture (Unpublished data).

Just recently, *B. mandrillaris* had never been isolated from CSF, however trophozoites has been found in the CSF rarely, if a wet mount of the CSF is examined immediately may reveal actively moving trophozoites. *B. mandrillaris* was isolated from the CSF from a patient who developed infection after receiving a kidney transplant [42, 50,51].

The disease is not exclusive to human kind but has been reported in a variety of animals including gorillas, baboons, gibbons, monkeys, horses, sheep and dogs [52-55].

DESCRIPTION FROM PERUVIAN CASES

Cutaneous Disease and CNS Disease

At the University Hospital Cayetano Heredia in Lima, Peru have been 55 cases of free-living amoebiasis (FLA) since 1985. The first report sare findings of 10 autopsies from 12 patients of the series which death usually occurred within a few days or weeks [56]. In a review of the

55 cases studied at Cayetano Heredia University in Lima, Peru; 20 cases have been confirmed by immunofluorescence testing as having been caused by *Balamuthia mandrillaris* [57,41]. All patients initially presented with a cutaneous lesion, allowing a diagnosis to be made while they were alive. The typical presentation is a painless, nonulcerated plaque on the central portion of the face-usually the nose-and less often on other areas such as the mandible, abdomen, and extremities (Figure. 9). The consistency varies from slightly rubbery to rock-hard. Histopathologic findings include a diffuse lymphoplasmocytic infiltrate with histiocytes, ill-defined granulomas, and numerous multinucleated giant cells occupying mainly the dermis and occasionally the subcutaneous fat. Amoebo-trophozoites are present in approximately 75% of cases. Unless a distinct protozoan nucleus and nucleolus are seen, the trophozoites can be easily confused with the cytoplasm of histiocytes, [41, 46,57].

Epidemiology in South America - Peru

The Infection with *B. mandrillaris* is now recognized as an emerging disease, and the parasite causes the often deadly BMAI. The clinical manifestations of patients infected with either *Acanthamoeba* spp. or *B. mandrillaris* are similar and will be considered together.

Fatal infections due to *B. mandrillaris* have more than 200 cases have been reported worldwide. The ages of people infected by this amoeba spans from 1 to 89 years old, which makes difficult to assess the predisposing behaviors to this infection. However, 55% of the cases have been reported in people younger than 15 years old and the Hispanic background was found in 45% of the total reported cases [41,58,59] In a 2011 review of 35 cases in the United States, most came from the southern states: 15 from California, 6 in Arizona, 6 in Texas, and 3 from Mississippi [59]. Inexplicably, in the thesis published by Mosser 2011. it is shown that a lot of the data on ethnicity was lost [60].

B. mandrillaris has been reported in several Latin-American countries, namely Argentina [61], Brazil [62-65], Chile [66-67] Peru [41,57,58,68 -72], and México [73] From these countries, Peru has the highest incidence of infections accounting for 55 accumulated cases since 1975.

The number of Peruvian cases is higher in comparison to other countries (i.e. Mexico has accumulated only 13 cases in total), and shows the following statistics: frequency of infection by this amoeba in males is 2.5 times higher than in females and the registered cases seems to be localized in four departments Piura, Trujillo (Peruvian north coast), Lima and Ica. [15,46,57].

How do we make the Diagnosis in Peru?

Clinical presentation

Two main clinical presentations have been identified; the first is depicted by initial skin lesions followed by the development of neurologic manifestations in weeks to months.

The diagnosis of GAE will be very difficult and the prognosis very disastrous if the patient debuts with CNS involvement. On the contrary, if the patients present first with a skin lesion first, there is, at least in theory, the possibility of an earlier diagnosis and cure [41,46].

The lesion and biopsy (Histology)

The skin biopsy may serve as the first clue to an amebic infection. The description of the skin lesion is very important because that description may proceed to biopsy and an experienced pathologist is able to detect amoebae in tissue quickly. The most common location is the central face, especially on the nose (in Peru), . This lesion's borders are poorly defined although occasionally a raised border may give an idea of an annular lesion[41,46,56]. The diameter may vary from one to several centimeters; the color may vary from a skin tone to slightly red to a violaceous hue. On palpation they feel firm or rubbery [56,57]. The lesion with ulceration may occur, but it is more common in large lesions. These are more common in the legs and arms. The lesion is always asymptomatic, and the sensation is preserved.

The lesion may precede by weeks, months or years the CNS manifestations, and may disappear with treatment, but that's not always the case.

Most patients with cutaneous lesions seen at the IMTAvH are otherwise asymptomatic, and systemic symptoms, such as fever, malaise, anorexia or weight loss are absent [41,46,56,58,69]. In a lot of time it has been said that they are immuno-competent patients, but the truth is that there is no such evidence. The time and the immune conditions in which the amoeba enters the host is unknown. This could be the key to the infection.

The Amoeba

At microscopic examination with hematoxylin-eosin (H-E) staining, amebic trophozoites are often demonstrated within the infected parenchymal tissue and perivascular spaces. Trophozoites range in size from approximately 12 to 60 mm and typically contain a round nucleus with a large spherical nucleolus.

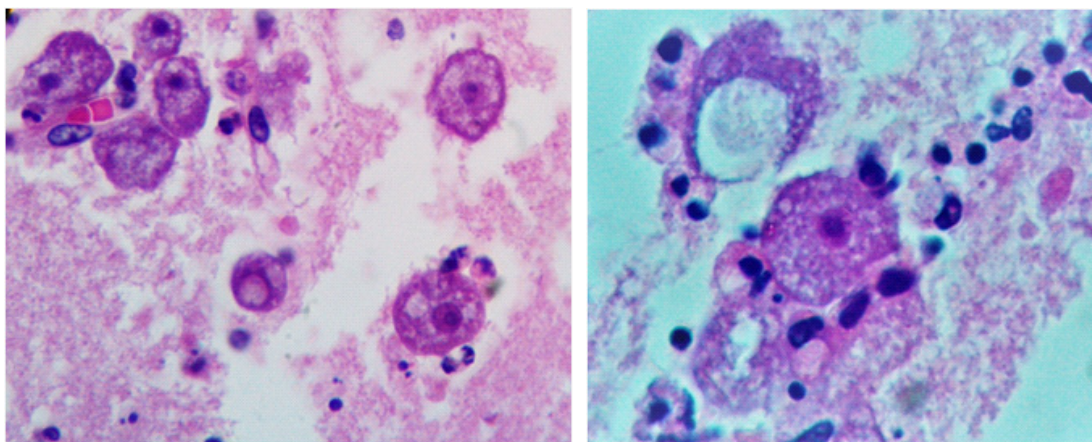


Figure N° 8: Histological image of a brain injury of a Peruvian patient. (H-E smears). Trophozoite of free living amoebae. [Photo, courtesy from Ma Carmen Garaycochea MSc.]

Amebic cysts ranging from 6 to 30 μm may be found and are best verified with Gomorimethenamine-silver stain or periodic acid-Schiff stain [74,75].

The morphology of the amoeba in fixed tissue is evident; nevertheless it is often difficult to find it. In Peruvian reports, the size of the amoebas was 30 to 40 μm in diameter, the cytoplasm was pink and finely granular with H&E, red to purple with PAS, and granular, faintly blue with trichrome. Silver Grocott stain is negative and Gomori is slightly positive. Most amoebae had a single nucleus but some had two nuclei. The nuclei measured, 10 to 12 μm in diameter with a prominent nucleolus. [46,57,58,71].

Histopathology

Brain biopsy typically demonstrates granulomas with foamy macrophages and multinucleated giant cells accompanied by lymphocytes. Amebae have a predilection for location in the vascular walls of capillaries and venules, and vascular damage may be present as tissue hemorrhage. Neutrophils may predominate and amebas may be seen [46].

Histopathological examination of skin biopsies stained with hematoxylin – eosin [56,57] allows the visualization of amoeba infiltrating the reticular dermis together with lymphocytes, plasma cells and histiocytes [56,57,58]. The latter can be distributed either diffusely or forming ill-defined granulomas. Nasal lesions shows aggregates of eosinophils, neutrophils and nuclear dust, while the amoebae may be seen as trophozoites.

in as much as two thirds of the tissue, but can be easily taken as histiocytes, although the amoeba cytoplasm may take a bubbly appearance [56,57]. Cerebral hemispheres, cerebellum and brainstem may show focal and isolated hemorrhagic softening associated to edema, slightly overlaying the surface of the convexity. The leptomeninges would be congested, opaque and adhering to cerebral cortex as described in the cases due to *Acanthamoeba* [56,57,76]. There have been also necrotic hemorrhagic areas in the brain and few cases also showed tonsil herniation. High numbers of amoebae have been seen infiltrating the subadventitial space of arteries, veins and capillary vessels leading to amoebic perivasculitis, capillary thrombosis and microinfarcts over the brain tissue [56, 57,58,76].

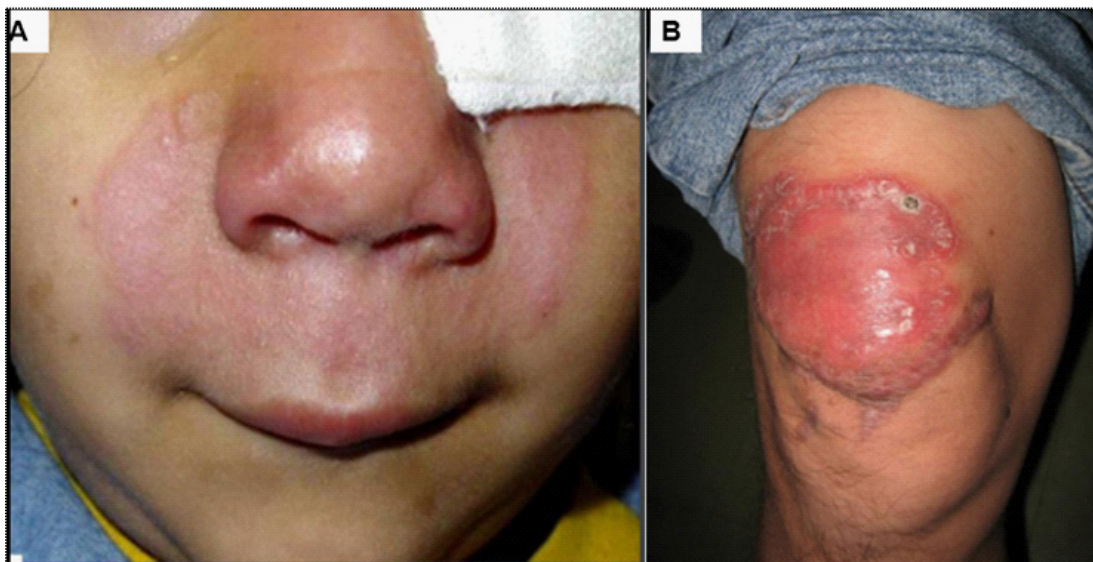


Figure N° 9: A) Central-facial skin lesion, morphological feature of *Balamuthia mandrillaris*. [Cortesy of IMT-AvH-UPCH. Lima-Peru.]: B) Skin plaque on the knee, very suggestive of *Balamuthia mandrillaris* infection. (Picture from Universidad Peruana Cayetano Heredia).

MOLECULAR BIOLOGY

Molecular techniques have been developed to identify *Balamuthia mnadrillaris* DNA in the CSF, skin lesion, brain tissue, and even in the environment [14,77]. The PCR amplifying the large fragment of the mitochondrial 16S rRNA gene was validated with brain tissue and CSF samples from suspected GAE patients and these studies have been successfully done at the CDC with high sensitivity in many samples.

Specific primers have been developed by Yagi et al., 2008 [78] to retrospectively confirm GAE in archivedslide specimens fixed in formalin and embedded paraffin. Additionally, a real-time multiplex PCR assay has been developed that can specifically identify *Balamuthia* DNA in human CSF and brain tissue in a short time [79].

In many cases, paraffin-shaped tissues do not allow large DNA replications, for this reason a modified version of this assay was developed , featuring a new reverse primer that resulted in a 230 pb amplicon [52].

The analysis from sequencing of nuclear 18S (**nDNA**) and mitochondrial 16S (**mDNA**) DNAs of *Balamuthia* isolated and found that the nDNA of four isolated ones was identical and the mDNA of seven isolates ones varied only to a maximum of 1.8%. The conclusions are that all the isolated of *Balamuthia* was of a single species with a worldwide distribution [77].

In Peru, we performed molecular identification of *Balamuthia mandrillaris* in samples of human and environmental origin, obtaining positive results [68]. However, we in Peru don't have the strain isolate axenic, necessary to isolate it from human sample and perform the genomic comparison.

Table N°4: Primer and probefor specific detection of *Balamuthia mandrillaris*.

PCR assay	oligonucleotide	Sequence (5' - 3')	Amplicon Size (bp)	Detection mode	Reference
1	5'Balspec16S	(5-CGCATGTATGAAGAAGACCA-3)	1,075 pb	Gel agarose	28
	3'Balspec16S	(5-TTACCTATATAATTGTCGATACCA-3)			
2	5Balspec16S	(5-CGCATGTATGAAGAAGACCA-3)	230	Gel agarose	80
	Bal16Sr610	(5-CCCCTTTTAACTCTAGTCATATAGT-3),			
3	BalaF1451	(5'-TAA CCT GCT AAA TAG TCA TGC CAA T-3')	171	fluorescence	79
	BalaR1621	(5'-CAA ACT TCC CTC GGC TAA TCA-3')			
	BalaP1582	(R) AG TAC TTC TAC CAA TCC AAC CGC CA (Q)			
4	RNase P/FW	(5'-GGC AGG TTC CGA GGA GAC A-3')	82	fluorescence	81
	RNase P/RV	(5'-GTG GCC TTG TGT ATT GAA CTT AAC ATT-3')			
	RNaseP/Probe	(R)-TGG AACCAT ACCTTG GGTGACACG ATG (Q)			
5	rps3-F	(5'-CTGYTCGATTTT CGAAAAATAAAGTAG-3')	1,290 bp	Gel agarose	51
	rps3-R	(5'-TGAAAGAAGAACATTAGATCACGACT-3')			

In our laboratory of Protozoarios y Endosimbiontes Patogenos at the IMT Alexander von Humboldt Lima - Peru, we have performed diagnostic tests for free-living amoebas. Among them, we have performed molecular studies for the diagnosis of *Balamuthia* in fresh skin and brain tissue (Manuscript in 2017).

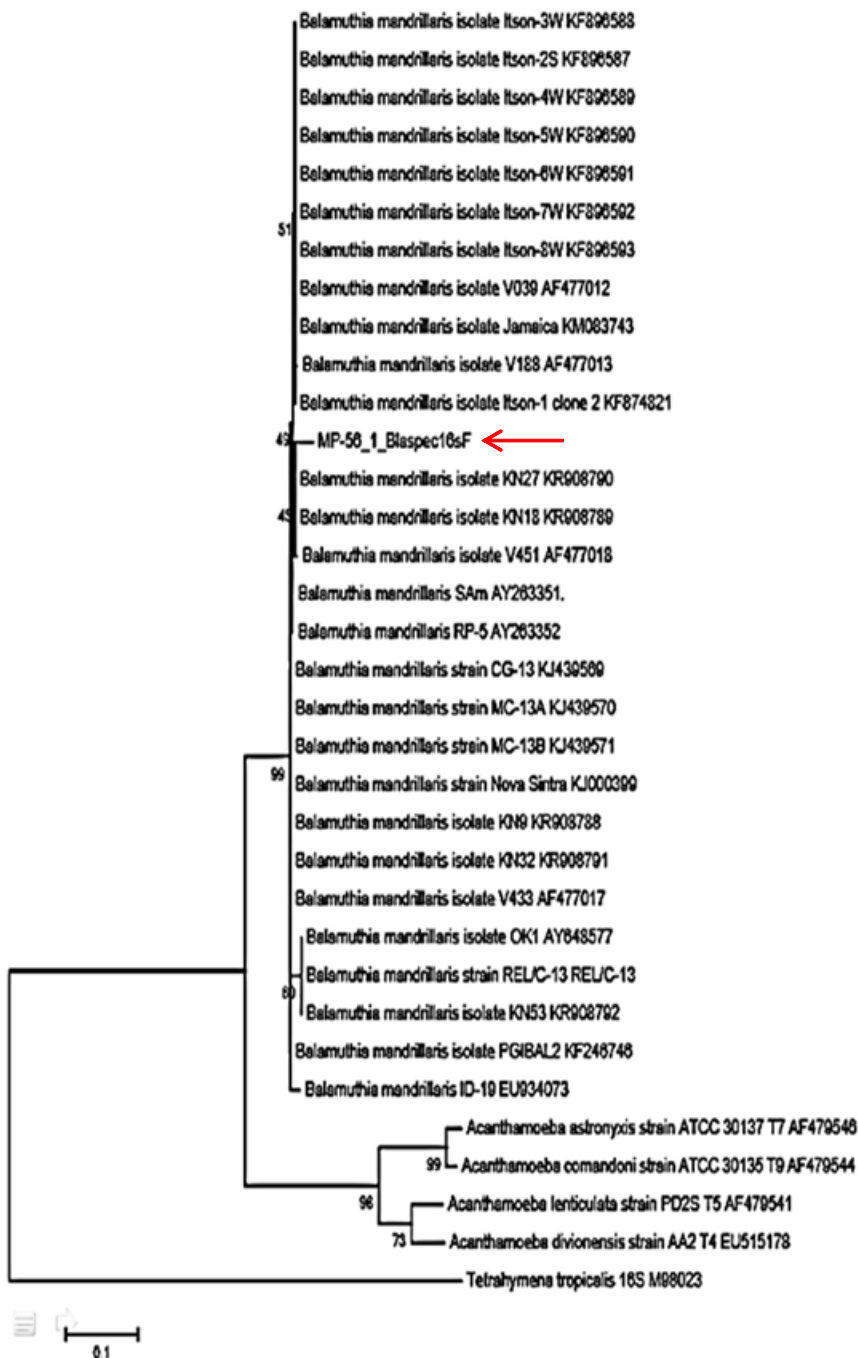


Figure N° 10: Phylogenetic relationships for the sequence of the mitochondrial small subunit rRNA gene between the isolates of *B. mandrillaris* identified in this study and previous isolates of *B. mandrillaris* deposited in Genbank. The arrow red identification from strain of *Balamuthia mandrillaris* from patient who die in 2015.

EFFECTIVE THERAPY

There is no effective treatment for BMAI and the prognosis is poor; low incidence, non-specific findings, delayed diagnosis and the lack of an model animal have all are virtually impossible studies in this moment. There are reports of 10 survivors within the medical literature, among 200 cases or more of *Balamuthia mandrillaris* infection worldwide. Although several patients are known to have survived because of diagnosis antemortem, BMAI is most often identified only at death [74]. In Peru, six surviving patients have been treated with a combination of fluconazole or itraconazole, in addition to albendazole and miltefosine [46]. Three patients had CNS involvement at the time of presentation, and three more had cutaneous involvement only. In some Peruvian cases there have been a wide resection of the skin lesion has been used as an additional therapy [46,68,82,83,84]. One of them had facial center lesion, this patient did not develop cerebral lesion after 3 months of treatment [87]. A similar approach may be useful for patients with single CNS lesions, followed by medical therapy [85].

- **Microbiological investigation on the high prevalence of infection of free living amoeba how *Balamuthia mandrillaris* and others amoeba *insertaesis* on the coast of Peru:**

Peru is the second country to report cases by *Balamuthia* worldwide and the first in Latin America. The reports in Peru are basically clinical and there are very few are the laboratory studies [85].

Meningoencephalitis by *Balamuthia mandrillaris* (GAE) has been generally reviewed from a clinical standpoint. The pioneers in registering the case series with skin lesion and subsequent neurological lesion in the IMT Alexander von Humboldt have been the Doctors: **Eduardo Gotuzzo MD** and **Francisco Bravo MD**. Alsomany other clinicians have contributed to learning more about this disease.

But, unfortunately not all cases are reported and / or published. In the last years 2000-2015 the number of cases increased. Both clinical presentations have been observed: with cutaneous lesion and without cutaneous lesion [84,86]. It has been established that the number of cases of GAE per year is approximately 1-2 cases. However, it is possible that the number of these cases is underestimated due to the time it takes the disease to manifest, either with the appearance of cutaneous lesions or neurological compromise. It is very likely that this condition may explain some of case previously described as lethal mid-line granuloma in Peru.

It is a priority in our Latin American region the availability of specialized laboratories in diagnosing these parasitic entities such as free-living amoebae. This has led us to develop a new laboratory for the clinical diagnosis of *Balamuthia mandrillaris* and *Acanthamoeba* sp T-4 generally, in addition to *Naegleria fowleri* and other new free-living amoebas that are appearing spontaneously.

References

1. Visvesvara GS, Martinez AJ, Schuster FL, Leitch GJ, Wallace SV, et al. Leptomyxid ameba, a new agent of amebic meningoencephalitis in humans and animals. *J Clin Microbiol*. 1990; 28: 2750-2756.
2. Visvesvara GS, Schuster FL, Martinez AJ. Balamuthia mandrillaris, N. G., N. Sp., agent of amebic meningoencephalitis in humans and other animals. *J Eukaryot Microbiol*. 1993; 40: 504-514.
3. Dongyou Liu. Molecular Detection of Human Parasitic Pathogens. Balamuthia Govinda S. Visvesvara and Yvonne Qvarnstrom. 2013.
4. Visvesvara GS, Maguire JH . Pathogenic and opportunistic free-living amebas: Acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri and Sappinia diploidea. In: RL Guerrant, DH Walker, PF Weller (Eds.), Tropical Infectious Diseases: Principles, Pathogens & Practice. 2006; 1114-1125.
5. Schuster FL, Visvesvara GS. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *Int J Parasitol*. 2004; 34: 1001-1027.
6. Visvesvara GS. Pathogenic and opportunistic freelifing amebae. In: PR Murray, EJ Baron (Eds.), Manual of Clinical Microbiology. 2007. 2082-2091.
7. Amaral Zettler LA, Nerad TA, O'Kelly CJ, Peglar MT, Gillevet PM, et al. A molecular reassessment of the Leptomyxid amoebae. *Protist*. 2000; 151: 275-282.
8. Booton GC, Carmichael JR, Visvesvara GS, Byers TJ, Fuerst PA. Genotyping of Balamuthia mandrillaris based on nuclear 18S and mitochondrial 16S rRNA genes. *Am J Trop Med Hyg*. 2003; 68: 65-69.
9. Adl SM, Simpson AG, Farmer MA, Andersen RA, Anderson OR, et al. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J Eukaryot Microbiol*. 2005; 52: 399-451.
10. Dunnebacke TH, Schuster FL, Yagi S, Booton GC. Balamuthia mandrillaris from soil samples. *Microbiology*. 2004; 150: 2837-2842.
11. Latifi AR, Niyati M, Lorenzo-Morales J, Haghighi A, Seyyed Tabaei SJ, et al. Presence of Balamuthia mandrillaris in hot springs from Mazandaran province, northern Iran. *Epidemiol Infect*. 2016; 144: 2456-2461.
12. Dunnebacke TH, Schuster FL, Yagi S, Booton GC. Isolation of Balamuthia amebas from the environment. *J Eukaryot Microbiol*. 2003; 50: 510-511.
13. Niyati M, Lorenzo-Morales J, Rezaeian M, Martin-Navarro CM, Haghi AM, et al. Isolation of Balamuthia mandrillaris from urban dust, free of known infectious involvement. *Parasitol*. 2009; 106: 279-281.
14. Ahmad AF, Andrew PW, Kilvington S. Development of a nested PCR for environmental detection of the pathogenic free-living amoeba Balamuthia mandrillaris. *J Eukaryot Microbiol*. 2011; 58: 269-271.
15. Cabello-Vilchez AM, Rodríguez-Zaragoza S, Piñero J, Valladares B, Lorenzo-Morales J. Balamuthia mandrillaris in South America: an emerging potential hidden pathogen in Perú. *Exp. Parasitol*. 2014. 145: S10-S19.
16. Lares-Jiménez LF, Booton GC, Lares-Villa F, Velázquez-Contreras CA, Fuerst PA. Genetic analysis among environmental strains of Balamuthia mandrillaris recovered from an artificial lagoon and from soil in Sonora, México. *Exp. Parasitol*. 2014; 145: S57-S61.
17. Retana-Moreira L, Abrahams-Sandí E, Cabello-Vilchez AM, Reyes-Batlle M, Valladares B, et al. Isolation and molecular characterization of Acanthamoeba and Balamuthia mandrillaris from combination shower units in Costa Rica. *Parasitol. Res*. 2014; 113: 4117-4122.
18. Todd CD, Reyes-Batlle M, Piñero JE, Martínez-Carretero E, Valladares B, et al. Balamuthia mandrillaris therapeutic mud bath in Jamaica. *Epidemiol Infect*. 2015; 143: 2245-2248.
19. Baquero RA, Reyes-Batlle M, Nicola GG, Martín-Navarro CM, López-Arencibia A, et al. Presence of potentially pathogenic free-living amoebae strains from well water samples in Guinea-Bissau. *Pathog. Glob. Health*. 2014; 108: 206-211.
20. Niyati M, Karamati SA, Lorenzo-Morales J, Lasjerdi Z. Isolation of Balamuthia mandrillaris from soil samples in North-Western Iran. *Parasitol*. 2015; 106: 279-281.
21. Tapia JL, Torres BN, Visvesvara GS. Balamuthia mandrillaris: in vitro interactions with selected protozoa and algae. *J Eukaryot Microbiol*. 2013; 60: 448-454.
22. Cabello-Vilchez AM, Reyes-Batlle M, Montalbán-Sandoval E, Martín-Navarro CM, López-Arencibia A, et al. The isolation of Balamuthia mandrillaris from environmental sources from Peru. *Parasitol Res*. 2014; 113: 2509-2511.
23. Jayasekera S, Sissons J, Tucker J, Rogers C, Nolder D, Warhurst D, et al. Post-mortem culture of Balamuthia mandrillaris from the brain and cerebrospinal fluid of a case of granulomatous amoebic meningoencephalitis, using human brain microvascular endothelial cells. *J Med Microbiol*. 2004; 53: 1007-1012.

24. Cerva L. Amoebic meningoencephalitis: axenic culture of *Naegleria*. *Science*. 1969; 163: 576.
25. Schuster FL, Visvesvara GS. Axenic growth and drug sensitivity studies of *Balamuthia mandrillaris*, an agent of amoebic meningoencephalitis in humans and other animals. *J Clin Microbiol*. 1996; 34: 385-388.
26. Kiderlen AF, Tata PS, Ozel M, Laube U, Radam E, et al. Cytopathogenicity of *Balamuthia mandrillaris*, an opportunistic causative agent of granulomatous amoebic encephalitis. *J Eukaryot Microbiol*. 2006; 53: 456-463.
27. Lares-Jiménez LF, Gámez-Gutiérrez RA, Lares-Villa F. Novel culture medium for the axenic growth of *Balamuthia mandrillaris*. *Diagn Microbiol Infect Dis*. 2015; 82: 286-288.
28. Schuster FL, Dunnebacke TH, Booton GC, Yagi S, Kohlmeier CK, et al. Environmental isolation of *Balamuthia mandrillaris* associated with a case of amoebic encephalitis. *J Clin Microbiol*. 2003; 41: 3175-3180.
29. Lares-Jiménez LF, Booton GC, Lares-Villa F, Velázquez-Contreras CA, Fuerst PA. Genetic analysis among environmental strains of *Balamuthia mandrillaris* recovered from an artificial lagoon and from soil in Sonora, Mexico. *Exp Parasitol*. 2014; 145: S57-S61.
30. Neidig N, Jousset A, Nunes F, Bonkowski M, Paul R.J, et al. Interference between bacterial feeding nematodes and amoebae relies on innate and inducible mutual toxicity. *Funct. Ecol*. 2010; 24: 1133-1138.
31. Adl, M.S., Gupta, V.V.S.R. Protists in soil ecology and forest nutrient cycling. *Can. J. For. Res*. 2006; 36: 1805-1817.
32. Groscop JA, Brent MM. The Effects of Selected Strains of Pigmented Microorganisms on Small Free-Living Amoebae. *Can J Microbiol*. 1964; 10: 579-584.
33. Rassmuson E.M, Carpentier T.C. Variations in tropical sea surface temperature and surface wind fields associated with the Southern Oscillation/El Niño. *Mon. Weather Rev*. 1982; 110: 354-384.
34. Waylen P.R, Caviedes C. El Niño and annual floods on the north Peruvian littoral. *J. Hydrol*. 1986; 89: 141-156.
35. Quinn W.H, Zopf D.O, Short K.S, Yang, R.T.W.K. Historical trends and statistics of the Southern oscillation, El Niño, and Indonesian droughts. *Fish. Bull*. 1978; 76: 663-668.
36. Matin A, Siddiqui R, Jayasekera S, Khan NA. Increasing importance of *Balamuthia mandrillaris*. *Lin Microbiol Rev*. 2008; 21: 435-448.
37. Lorenzo-Morales J, Cabello-Vilchez AM, Martín-Navarro CM, Martínez-Carretero E, Piñero JE, et al. Is *Balamuthia mandrillaris* a public health concern worldwide? *Trends Parasitol*. 2013; 29: 483-488.
38. Matin A, Stins M, Kim KS, Khan NA. *Balamuthia mandrillaris* exhibits metalloprotease activities. *FEMS Immunol Med Microbiol*. 2006; 47: 83-91.
39. Matin A, Siddiqui R, Jung SY, Kim KS, Stins M, et al. *Balamuthia mandrillaris* interactions with human brain microvascular endothelial cells in vitro. *J Med Microbiol*. 2007; 56: 1110-1115.
40. Cabello-Vilchez AM, Martín-Navarro CM, Lopez-Arencibia A, Reyes-Batlle M, Gonzalez AC, et al. Genotyping of potentially pathogenic *Acanthamoeba* strains isolated from nasal swabs of healthy individuals in Peru. *Acta Trop*. 2014; 130: 7-10.
41. Bravo FG, Seas C. *Balamuthia mandrillaris* amoebic encephalitis: an emerging parasitic infection. *Curr Infect Dis Rep*. 2012; 14: 391-396.
42. Jayasekera S, Matin A, Sissons J, Maghsood AH, Khan NA. *Balamuthia mandrillaris* stimulates interleukin-6 release in primary human brain microvascular endothelial cells via a phosphatidylinositol 3-kinase-dependent pathway. *Microbes Infect*. 2005; 7: 1345-1351.
43. Rocha-Azevedo B, Jamerson M, Cabral GA, Silva-Filho FC, Marciano-Cabral F. The interaction between the amoeba *Balamuthia mandrillaris* and extracellular matrix glycoproteins in vitro. *Parasitology*. 2007; 134: 51-58.
44. Schuster FL, Visvesvara GS. *Balamuthia mandrillaris*. *Emerging Protozoan Pathogens*. 2008; 71-118.
45. Matin A, Stins M, Kim KS, Khan NA. *Balamuthia mandrillaris* exhibits metalloprotease activities. *FEMS Immunol Med Microbiol*. 2006; 47: 83-91.
46. Seas C, Bravo F. Free-living amebas. 2015.
47. Intalapaporn P, Suankratay C, Shuangshoti S, Phantumchinda K, Keelawat S, et al. *Balamuthia mandrillaris* meningoencephalitis: the first case in southeast Asia. *Am J Trop Med Hyg*. 2004; 70: 666-669.
48. Doyle JS, Campbell E, Fuller A, Spelman DW, Cameron R, et al. *Balamuthia mandrillaris* brain abscess successfully treated with complete surgical excision and prolonged combination antimicrobial therapy. *J Neurosurg*. 2011; 114: 458-462.
49. Botterill E, Yip G. A rare survivor of *Balamuthia* granulomatous encephalitis. *Clin Neurol Neurosurg*. 2011; 113: 499-502.

50. Centers for Disease Control and Prevention (CDC). Balamuthia mandrillaris transmitted through organ transplantation-Mississippi. *Morb Mort Wkly Rep*. 2010; 59: 1165-1170.
51. Greninger AL, Messacar K, Dunnebacke T, Naccache SN, Federman S, et al. Clinical metagenomic identification of Balamuthia mandrillaris encephalitis and assembly of the draft genome: the continuing case for reference genome sequencing. *Genome Med*. 2015; 7: 113.
52. Foreman O, Sykes J, Ball L, Yang N, De Cock H. Disseminated infection with Balamuthia mandrillaris in a dog. *Vet Pathol*. 2004; 41: 506-510.
53. Finnin PJ, Visvesvara GS, Campbell BE, Fry DR, Gasser RB. Multifocal Balamuthia mandrillaris infection in a dog in Australia. *Parasitol Res*. 2007; 100: 423-426.
54. Gjeltema JL, Troan B, Muehlenbachs A, Liu L, Da Silva AJ, et al. Amoebic meningoencephalitis and disseminated infection caused by Balamuthia mandrillaris in a Western lowland gorilla (*Gorilla gorilla gorilla*). *J Am Vet Med Assoc*. 2016; 248: 315-321.
55. Visvesvara GS, Moura H, Schuster FL. Pathogenic and opportunistic freeliving amoebae: Acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri, and Sappiniadiplodea. *FEMS Immunol Med Microbiol* 2007; 50: 1-26.
56. Campos P, Cabrera J, Gotuzzo E, Guillén D. Neurological involvement in free living amebiasis. *Rev Neurol*. 1999; 29: 316-318.
57. Bravo F.G, Cabrera J, Gotuzzo E. Cutaneous manifestations of infection by free living amebas, with special emphasis on Balamuthia mandrillaris. In: Tyring, S.K., Lupi, O., Henage, U.R. (Eds.), *Tropical Dermatology*. 2006; 49-56.
58. Bravo FG, Alvarez PJ, Gotuzzo E. Balamuthia mandrillaris infection of the skin and central nervous system: an emerging disease of concern to many specialties in medicine. *Curr Opin Infect Dis*. 2011; 24: 112-117.
59. Diaz JH. The public health threat from Balamuthia mandrillaris in the southern United States. *J La State Med Soc*. 2011; 163: 197-204.
60. Moser MA. A Descriptive Review of Balamuthia and Non-Keratitis Acanthamoeba Cases in the United States. 2011.
61. Taratuto AL, Monges J, Acefe JC, Meli F, Paredes A, et al. Leptomyxid amoeba encephalitis: report of the first case in Argentina. *Trans R Soc Trop Med Hyg*. 1991; 85: 77.
62. Chimelli L, Hahn MD, Scaravilli F, Wallace S, Visvesvara GS. Granulomatous amoebic encephalitis due to leptomyxid amoebae: report of the first Brazilian case. *Trans R Soc Trop Med Hyg*. 1992; 86: 635.
63. Rodríguez R, Méndez O, Molina O, Luzardo G, Martínez AJ, et al. Central nervous system infection by free-living amebas: report of 3 Venezuelan cases. *Rev Neurol*. 1998; 26: 1005-1008.
64. Silva-Vergara M.L, Da Cunha Colombo E.R, De Figueiredo Vissotto E, Silva A.C, Chica J.E, et al. Disseminated Balamuthia mandrillaris amoeba infection in an AIDS patient from Brazil. *Am. J. Trop. Med. Hyg*. 2007; 77: 1096-1098.
65. Silva RA, Araújo Sde A, Avellar IF, Pittella JE, Oliveira JT, et al. Granulomatous amoebic meningoencephalitis in an immunocompetent patient. *Arch Neurol*. 2010; 67: 1516-1520.
66. Oddó B D, Ciani A S, Vial C P. Granulomatous amebic encephalitis caused by Balamuthia mandrillaris. First case diagnosed in Chile. *Rev Chilena Infectol*. 2006; 23: 232-236.
67. Cuevas P M, Smoje P G, Jofré M L, Ledermann D W, Noemí H I, et al. Granulomatous amoebic meningoencephalitis by Balamuthia mandrillaris: case report and literature review. *Rev Chilena Infectol*. 2006; 23: 237-242.
68. Martínez DY, Seas C, Bravo F, Legua P, Ramos C, et al. Successful treatment of Balamuthia mandrillaris amoebic infection with extensive neurological and cutaneous involvement. *Clin Infect Dis*. 2010; 51: e7-e11.
69. Valverde J, Arrese JE, Piérard GE. Granulomatous cutaneous centrofacial and meningocerebral amebiasis. *Am J Clin Dermatol*. 2006; 7: 267-269.
70. Gotuzzo E, Bravo F, Cabrera J. Amebas de vida Livre- Enfase na Infecção por Balamuthia mandrillaris. In: Cimerman, S., Cimerman, B. (Eds.), *Medicina Tropical*. 2003; 59-64.
71. Recavarren-Arce S, Velarde C, Gotuzzo E, Cabrera J. Amoeba angeitic lesions of the central nervous system in Balamuthia mandrillaris amebiasis. *Hum Pathol*. 1999; 30: 269-273.
72. Velarde C. Amebiasis de vida libre en el Perú: Un estudio anatomopatológico. 1996.
73. Riestra-Castaneda J.M, Riestra-Castaneda R, Gonzalez-Garrido A.A, Pena Moreno P, Martinez A.J, et al. Granulomatous amebic encephalitis due to Balamuthia mandrillaris (Leptomyxiidae): report of four cases from Mexico. *Am. J. Trop. Med. Hyg*. 1997; 56: 603-607.

74. Visvesvara GS, Balamuth W. Comparative studies on related free-living and pathogenic amoebae with special reference to *Acanthamoeba*. *J Protozool.* 1975; 22: 245-256.
75. Denney CF, Iragui VJ, Uber-Zak LD, Karpinski NC, Ziegler EJ, et al. Amebic meningoencephalitis caused by *Balamuthia mandrillaris*: case report and review. *Clin Infect Dis.* 1997; 25: 1354-1358.
76. Martinez A.J. Free-living Amebas: Natural History, Prevention, Diagnosis, Pathology and Treatment of Disease. 1985.
77. Booton GC, Carmichael JR, Visvesvara GS, Byers TJ, Fuerst PA. Identification of *Balamuthia mandrillaris* by PCR assay using the mitochondrial 16S rRNA gene as a target. *J Clin Microbiol.* 2003; 41: 453-455.
78. Yagi S, Schuster FL, Visvesvara GS. Demonstration of *Balamuthia* and *Acanthamoeba* mitochondrial DNA in sectioned archival brain and other tissues by the polymerase chain reaction. *ParasitolRes.* 2008; 102: 491-497.
79. Qvarnstrom Y, Visvesvara GS, Sriram R, da Silva AJ. Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. *J Clin Microbiol.* 2006; 44: 3589-3595.
80. Yagi S, Booton G.C, Visvesvara G.S, Schuster F.L. Detection of *Balamuthia* 16S rRNA gene DNA in clinical specimens by PCR. *J. Clin. Microbiol.* 2005; 43: 3192-3197.
81. Kiderlen AF, Radam E, Lewin A. Detection of *Balamuthia mandrillaris* DNA by real-time PCR targeting the RNase P gene. *BMC Microbiol.* 2008; 8: 210.
82. Martinez DY, F Bravo, E Gotuzzo. A novel therapeutic option for *Balamuthia mandrillaris* infection. 2012.
83. Orozco L, Hanigan W, Khan M, Fratkin J, Lee M. Neurosurgical intervention in the diagnosis and treatment of *Balamuthia mandrillaris* encephalitis. *J Neurosurg.* 2011; 115: 636-640.
84. Orrego-Puelles E, Casavilca S, Rodríguez FJ, Pritt BS, Castillo M, et al. Amebiasis of the central nervous system: report of six cases in Peru. *Rev Peru Med Exp Salud Publica.* 2015; 32: 591-597.
85. Cabello Vilchez AM, *Acanthamoeba* SP. Un Agente Oportunistas. En *Infecciones Humanas. Acanthamoeba* sp. An Opportunistic Agent Human Infections in Spanish. *Revista de Investigación de la Universidad Norbert Wiener.* 2015; 4: 11-32.
86. Cabello-Vilchez AM. *Balamuthia mandrillaris* en el Perú, lesiones cutáneas, meningoencefalitis y métodos de cultivo. 2016; 20: 107-119.
87. Bravo, F.G., Gotuzzo, E. Cutaneous manifestations of infection by free living amoebas, In: Tying, S.K., Lupi, O., Henage, U.R. (Eds.), 2 Ed. *Tropical Dermatology.* 2017; 50-55.