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Review articles

Amoebas from the genus *Acanthamoeba* and their pathogenic properties

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ABSTRACT. Amoebas from the genus *Acanthamoeba* are cosmopolitan organisms, which can exist as free-living organisms and as parasites within host tissue. *Acanthamoeba* infection present a serious risk to human health and are characterized by high mortality, especially in immunocompromised individuals. These protozoa are the etiological factors of granulomatous amoebic encephalitis (GAE) and *Acanthamoeba* keratitis (AK). They can also live in the lungs, adrenals glands, nose, throat, and bones of the host. Furthermore, the amoebas can be vectors of pathogenic bacteria. *Acanthamoeba* infection caused is a serious clinical problem mainly due to limited progress in diagnostics and treatment of this infection, which is associated with insufficient knowledge of pathogenesis, pathophysiology and the host immune response against *Acanthamoeba* antigens. This review study presents the biology of *Acanthamoeba* sp. as well as pathogenicity, diagnostics, and treatment of amoebas infections. It also presents data, including experimental results, concerning pathogenic properties and the host's immunology response against *Acanthamoeba* sp.

Key words: *Acanthamoeba* sp., diagnostics, treatment, pathogenesis, immunology response

Introduction

Amoebas from the genus *Acanthamoeba* pose a threat to human health and life, especially to persons with a lower immunity level. Due to the lack of appropriate diagnostic methods and effective treatment, *Acanthamoeba* infection are characterized by high mortality [1]. In addition, amoebas have the ability to transmit microbes that may show increased virulence and drug resistance [2]. There are many scientific studies on interactions between *Acanthamoeba* sp. and host. These studies are often conducted on animal models, mainly BALB/c and Swiss mice, but also on rats, hamsters, rabbits, pigs and locusts (*Locusta migratoria)* [3–6]. The pathomorphological and pathophysiological changes observed in these animals, especially in mice, are analogical to those found in humans. In addition, specific murine monoclonal antibodies are available commercially, which enables multidirectional studies [7].

Morphology of *Acanthamoeba* species

Acanthamoeba sp. are free-living protozoa that occur commonly in the natural environment. They are amphizoic organisms which can be either free-living or parasitic [8]. Amoebas can be found in trophozoite or cyst form. The trophozoite is 13–40 μm in diameter. The protozoa cells contains nucleus with central nucleolus, single pulsating vacuole and numerous digestive vacuoles. Spiny surface structures called acanthopodia or lobopodia are responsible for amoeboid movement [9]. After a period of intensive growth or as a result of unfavourable environmental conditions, trophozoite may transform into a cyst (encystation) which may retain its pathogenic properties even up to a dozen or so years [10,11]. Depending on the species, cysts have a round or polygonal shape and are surrounded by a double shell: the outer strongly pleated (ectocyst) and the smooth inner (endocyst) in a star-shaped or polygonal shape, which clearly protrudes from the outer shell in several places [12]. Cysts, whose diameter usually does not exceed 25 μm, are resistant to many physical and chemical factors. They can survive at low temperatures, are resistant to drying out, ultraviolet radiation, variable osmotic pressure, changes in humidity, changes in the concentration of hydrogen ions as well as organic and inorganic compounds [9]. It has been shown that all development forms of amoebas are invasive to humans [13].

Genotypes of *Acanthamoeba* species

Initially, the identification of *Acanthamoeba* sp. was based on morphological features, including the size, shape of ecto- and endocysts and temperature requirements necessary for the growth of the organisms. Currently, the identification is conducted with the use of biochemical and molecular biology techniques [9,14,15]. To date, 22 genotypes (T1-T22) have been identified, based on the analysis of the 18S rRNA gene sequence using the polymerase chain reaction (PCR) [16]. It was found that *Acanthamoeba* sp. of the genus T2-T6 and T10, T11, and T15 are the etiological factors of *Acanthamoeba* keratitis (AK), whereas the genotypes T1, T2, T4, T5, T10, and T12 are the factors of granulomatous amebic encephalitis (GAE) [17]. The latest identified genotypes include the environmental genotype T19 (*Acanthamoeba micheli*) [18] and the pathogenic genotype T20, which was initially classified as T4 [19] as well as genotypes T21 and T22 (T22 – *Acanthamoeba pyriformis).* However, genotypes T21 and T22 have not published in the database yet [20]. The most commonly isolated strain of *Acanthamoeba* from patients with GAE and AK is *Acanthamoeba* T4, which is suggested to be characterized by increased virulence and reduced sensitivity to chemotherapeutic agents [12].

Occurrence of *Acanthamoeba* species

Acanthamoeba sp. are cosmopolitan protozoa, which are ubiquitous in natural environment. Natural antibodies against *Acanthamoeba* IgG have been found in peripheral blood in over 80% of human population [21]. The amoebas were isolated from water, soil and air. They can be found in natural and artificial water reservoirs [22], as well as in dust, fans, air-conditioning, lens fluids and medical equipment, including dental units and dialysis stations [23–25]. They were also observed in sandboxes, fountains, communal sewage and tap water [25,26]. Amoeba strains were isolated from fruits, vegetables, plants, fungi and some animal species [22,27]. In addition, protozoa were isolated from biological materials, including bacterial cultures, gastric and intestinal lavage, cerebrospinal fluid (CSF), sputum, bronchoalveolar lavage (BAL), nasopharyngeal and fragments of liver, kidneys, spleen, lungs and corneal scrapings [8,28–30].

Pathogenic properties

Factors determining pathogenicity of amoebas were divided into direct and indirect factors [9]. The direct agents are the ability to adhere, phagocytosis, secretion of specific enzymes and acanthoporine which is cytotoxic to human neurons and antibacterial activity against various bacterial strains by increasing the permeability of their membranes [31]. In highly pathogenic amoebas were observed highly activity of proteolytic enzymes, mainly serine and cysteine proteases allowing *Acanthamoeba* sp. infection to the corneal stromal cornea with accompanying inflammatory reactions, oedema or necrosis [9,32]. Hadaś [33] observed that pathogenic strains of free-living amoeba were characterized by high activity of collagenase, elastase, as well as peroxidase, and low activity of superoxide dismutase. Correlations were also found between the activity of prostaglandin synthetase and induction of *Acanthamoeba* sp. infection, but no relationship between the activity of catalase and the virulence and infectiveness was noticed [33].

Indirect factors include the ability to encyste, morphology of protozoa, tolerance to changing environmental conditions, ubiquity, biofilms, chemotaxis, host health and drug resistance [9]. The pathogenicity of *Acanthamoeba* sp. trophozoites may be due to the number of acanthopodia that allow contact with host cells. Pathogenic amoebas have about 100 acanthopodia on their surface, and non-pathogenic ones have around 20 acanthopodia [32]. In addition, it was found that amoebas lacking these structures do not bind to epithelial cells of the cornea [10]. Pathogenic *Acanthamoeba* sp. show increased thermal tolerance compared to non-pathogenic strains [29]. Amoebas with pathogenic properties are able to grow and develop at 42°C and above, which is most likely due to their high levels of heat shock proteins (HSP60 and HSP70) [34].

The ability of *Acanthamoeba* sp. to grow at high temperature, osmolality and pH correlates with pathogenicity, as well as with their prevalence in various environments [32]. However, non-pathogenic strains with high thermal tolerance and non-thermophilic non-pathogenic strains have been described in the literature [32,35].

Amoebas are vectors of many pathogenic bacteria that facilitate their growth as well as development and protect microbes from adverse environmental conditions and chemical factors [27]. It was found that 25% of *Acanthamoeba* sp. isolated from the environment are bacterial vectors. Free-living amoebae can be a reservoir of pathogenic bacteria such as *Vibrio cholerae*, *Mycobacterium tuberculosis*, *Yersinia enterocolitica*, *Escherichia coli*, *Enterobacter cloacae*, *Listeria monocytogenes*, *Helicobacter pylori*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophila*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Salmonella enterica*, *Shigella dysenteriae*, *Staphylococcus aureus*. Apart from bacteria, amoebae can also transmit fungi, protozoa and viruses [27,36].

The immune response of the host

In the available scientific literature, there is little data on the host's immune response to *Acanthamoeba* sp. infection. Studies have shown that infection with free-living amoebas induces both innate and adaptive immune responses [1].

An important role of recognizing *Acanthamoeba* sp. infection and inducing cytokine production, including interleukin 8 (IL-8), tumour necrosis factor (TNF-α) and interferon beta (IFN-β) in the host's body, is played by Toll-like receptors (TLRs) [37]. They are mainly associated with cells of the immune system and they occur on monocytes, macrophages, dendritic cells, B lymphocytes, eosinophils, neutrophils and mast cells [38]. TLR2 and TLR4 receptors are the best-known TLRs [15]. Increased levels of TLR2 and TLR4 mRNA expression were found in the lungs and brains of mice infected with *Acanthamoeba* sp., which may suggest the effect of these receptors on the initiation of the immune response [15,39]. Alizadeh et al. [40] demonstrated that the TRL4 receptor is responsible for the recognition of *Acanthamoeba* sp. These authors also observed an increase in the expression of this receptor in the cornea of hamsters infected with amoeba. The possibility for the parasite to be recognized by TLR3 and TLR5, which are activated by double-stranded RNA and bacterial flagellin, respectively, is low because amoebae do not become intracellular and do not have flagellum [40].

The complement system is a barrier to many infections and works in a cascade system destroying bacteria and protozoa [41]. Activation of the complement system can occur either through the classic or alternative pathway, however the role of these proteins is not fully understood. *In vitro* studies with human serum showed that the complement and antibodies show lytic activity against *Acanthamoeba* sp. in the presence of phagocytes [42]. These reactions may stimulate the secretion of proinflammatory cytokines, including IL-1, IL-6 and TNF-α [43–45], which are released from monocytes and macrophages leading to the activation of neutrophils and vascular endothelial cells. It has been shown that TNF-α induces encystation of *Acanthamoeba* sp., which makes them resistant to phagocytosis [46]. Furthermore, it was found that trophozoites of pathogenic *Acanthamoeba* sp. are resistant to lysis through complementation due to the expression of regulatory proteins which exclude the complement cascade [47].

Phagocytic cells, such as neutrophils and macrophages, play a role in the destruction of *Acanthamoeba* sp. It has been found that macrophages may be involved in initiating and maintaining an effective immune response, but may also play a role in tissue repair processes [1]. *In vivo* studies concerning *Acanthamoeba* keratitis showed the presence of neutrophils in the brain and in the retina of the eye which may be involved in inhibiting the spread of invasion to other organs [48,49]. In mice infected with *Acanthamoeba* sp., a significant increase in natural killer (NK) cells was also observed, which suggests that these cells also participate in the protection of the body against these protozoa [50].

Animal studies have shown that subcutaneous immunization with trophozoites and cysts of *Acanthamoeba* sp. induces specific immunity in the form of delate type hypersensitivity and the production of IgG antibodies [3]. Recent studies on the AK mouse model showed that an infection of amoebas induces T helper (Th) lymphocytes and regulatory T lymphocytes (Treg) in the cornea and the atopic lymph nodes. In addition, the *Acanthamoeba* sp. infection induces the expression of IL-17A which plays an important role in relieving the symptoms of ocular infection [51].

The intensity of inflammation depends on the functionality of the immune system, which may be regulated by the increased expression of cyclooxygenase (COX) [52]. Important mediators of the pathophysiology in experimental acanthamoebosis occurred to be COX-1 and COX-2. *Acanthamoeba* sp. induced a expression of COX-1 and COX-2 proteins in the lungs of immunocompetent mice and a decrease in COX-1 and COX-2 in lung tissues in immunosuppressed *Acanthamoeba* sp. infected mice. However, it was also observed that expression of COX-1 and COX-2 proteins in the lungs of immunocompetent animals *Acanthamoeba* sp. infected does not correspond to differences in the expression of prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) [53].

Pathogenesis of *Acanthamoeba* infection

Acanthamoeba sp. infections pose a threat to the health and life of the hosts. The infection are characterized by relatively high mortality despite low incidence [54]. Amoebae can cause cerebral and extracerebral infections concerning the cornea, lungs, kidneys and skin. The infection may develop in immunocompetent as well as immunocompromised patients [55].

Granulomatous amoebic encephalitis (GAE)

Granulomatous amoebic encephalitis is an opportunistic disease with mortality around 97-98%. Infection mostly occurs in people with metabolic, physiological and immunological disorders caused by e.g. HIV infection, and in persons with organ transplants or chronic diseases such as diabetes [30,56]. The main site of penetration is the olfactory epithelium in the nasal cavity, and the infection occurs through air inhalation or aspiration of water contaminated with invasive forms of the protozoan [13]. Trophozoites migrate to the central nervous system through the nasal mucous membrane, the endothelium of the capillaries of the brain and the ethmoid bone along the olfactory nerves [57]. The site of the infection may also include the oral mucosa, damaged or ulcerative skin, ocular cornea and intestinal mucosa [29]. The pathogenesis of infection is not precisely understood. Infection is chronic or subacute, with focal necrotic lesions leading to death after 8 days to several months after the onset of the first symptoms [58]. Clinical symptoms associated with the presence of parasites in the brainstem or midbrain are psychiatric disorders, including confusion, lethargy and hallucinations. Patients with GAE also had headache, stiff neck, changes in body temperature, seizures and epileptic seizures, nausea and vomiting [59]. Clinical symptoms of GAE are not specific and the disease is often diagnosed as bacterial or viral encephalitis [27].

In the diagnosis of GAE, cerebrospinal fluid is used and lymphocytic pleocytosis, elevated protein levels and normal or reduced glucose are commonly observed [60]. Amoeba can also be isolated from the patient's tissues and grown *in vitro*. The material collected from the patient is placed on special culture media coated with inactivated bacteria, including *Escherichia coli* and *Enterobacter aerogenes*, with the plates subsequently incubated at 37°C [55]. Other methods used to identify *Acanthamoeba* sp. are immunodiagnostic and molecular methods, including PCR, real-time PCR and restriction fragment length polymorphism (RFLP) [9,61].

Treatment of brain infections caused by *Acanthamoeba* sp. is still difficult due to nonspecific symptoms and lack of diagnostic methods with high sensitivity and specificity. The treatment uses antibiotics used alone or combined therapy, including azole derivatives (fluconazole, ketoconazole, itraconazol, voriconazole), amphotericin B, rifampicin, pentamine, sulfadiazine and flucytosine [62].

The first studies of *Acanthamoeba* sp. pathogenicity on experimental animals were conducted by Culbertson et al. [63]. Amoebas isolated from tissue culture were administered to mice using intra cerebral and intravenous pathways. The study demonstrated extensive choroid plexus inflammation and meningitis as well as encephalomyelitis in the hosts [63]. Therefore, since the 1960s, various strains of laboratory mice have been used for *in vivo* experiments in experimental brain acanthamoebosis. Previous studies demonstrated the presence of the following neurological symptoms in experimentally infected mice: erratic running in circles, hyperactivity and convulsions [60]. Histological and morphological analysis of the brain showed oedema, decreasing of fissures and a strong meningeal congestion. In addition, detachment of cerebral meninges from the cortical part of the cerebral hemispheres mainly in the frontal lobe region was observed [64].

***Acanthamoeba* keratitis (AK)**

The structure of the human eye and direct exposure to environmental factors make it susceptible to many infections, including *Acanthamoeba* sp. [65]. The first cases of amoeba infection into the cornea were described in 1974 [66], and more than 3,000 cases of AK have been described since then [25]. Persons using contact lenses are at the highest risk of infection [27,67]. Currently, about 90% of patients diagnosed with AK are persons using contact lenses [9,29]. Another risk factor is previous mechanical cornea injury combined with exposure to contaminated water or soil [68]. The etiological factors of the AK include: *A. castellani*, *A. polyphaga*, *A. rhysodes*, *A. culbertsoni*, *A. hatchetti*, *A. lugdunesis*, *A. quin* and *A. griffini* [32,69].

The first stage of *Acanthamoeba* infection is the adhesion of amoeba to epithelial cells of the host cornea. The elements involved in this process are acanthopodia and adhesins, including mannose-binding protein (MBP) and laminin-binding proteins [32,70]. Amoebae then penetrate the corneal epithelium causing phagocytosis and secretion of toxins responsible for the induction of apoptosis [10,71,72]. Protozoa secrete neuraminidase which plays a role in the colonization of the parasite [32] and proteases which lead to the degradation of basal membranes [12]. The mannose-induced cytopathic protein (MIP-133) is one of the serine proteases inducing degradation of keratocytes, ciliary body iris cells, retinal pigment epithelium, corneal epithelium and corneal endothelial cells [32]. Proteases allow the *Acanthamoeba* sp. infection. Into the cornea stroma with associated inflammatory reactions, oedema and necrosis [32]. The apoptotic bodies, changes in the cell membrane, chromatin condensation and DNA fragmentation can be observed In the infected cells of the corneal epithelium [9,22]. The last stage of the infection is corneal nerve inflammation [73].

Symptoms of AK include severe eye pain, blurred vision, photophobia, redness, foreign body sensation, followed by oedema of the conjunctiva and eyelids [32,74]. The inflammation of the lacrimal gland, extraocular muscle inflammation and have also been described [75]. Characteristic symptoms of the AK are single or multiple annular infiltrates in the central part of the cornea combined with the disappearance of keratocytes [32,65]. These changes usually occur within one eye, but cases of bilateral invasion have also been described [76]. Advanced changes in the cornea can even lead to vision loss. Quick diagnostics and undertaking appropriate, long-term treatment allows to restore the proper functioning of the eye [77].

Acanthamoeba keratitis diagnosis is based on examination of corneal scrapings or material taken during corneal biopsies using haematoxylin and eosin staining, confocal microscopy and immunofluorescence methods [78,79]. It is not recommended to take swabs from the eyeball or tears, due to the rapid amoeba penetration into the subsequent layers of the cornea. In addition, similarly to GAE, breeding techniques and molecular methods are used [78,79].

Acanthamoeba keratitis treatment is difficult and long-lasting [80]. Propidium (0.1%), hexamidine, chlorhexidine (0.02%), neomycin, paromomycin, polynxin B, clotrimazole and itraconazole are used. Steroids are also used to relieve pain and reduce inflammation [27,81]. A recent report has demonstrated the effect of lactoferrin, an antimicrobial glycoprotein, on the survival and encystication of trophozoite of *Acanthamoeba* sp. which causes AK [82]. It was found that iron-free bovine milk lactoferrin (apo-bLF) has a potent amoebidical effect on trophozoites, in contrast to iron-saturated bovine milk lactoferrin (Fe-bLF). Following the incubation of *Acanthamoeba* sp. with apo-bLF, most of the dead cells were found to be non-spherical amoeba trophozoites. On the other hand, apo-bLF did not show any amoebidical effect on spherical trophozoites or cysts [82]. However, an *in vivo* study on mice by Hadaś et al. [83] found that tea tree oil can be used in the treatment of AK as it affects both trophozoites and cysts of *Acanthamoeba* sp. In addition, the study showed that the tea oil did not damage the cornea of the studied animals [83].

Most experimental studies on *Acanthamoeba* sp. infection are carried out on mice. However, Ren and Wu [7] found that it is preferable to study *Acanthamoeaba* keratitis on rats than on mice. Although the amoebas penetrate the murine cornea more quickly than the rat cornea, the body size, level of immune response and limited range of anaesthetic doses increased the risk of death of mice [7]. However, recent reports indicate that the best model for experimental AK research is rabbit, as the morphological and anatomical structure of rabbit cornea is more similar to human cornea [84]. *Acanthamoeba* keratitis is induced in rabbits by amoeba microinjection to the area between the corneal epithelium and Bowman's membrane. In contrast, amoeba injection into the stroma of corneal leads to a rapid inflammatory reaction, and in some animals can cause severe encephalitis [6].

Acanthamoeba sp. infection to other organs and tissues

Infections caused by the *Acanthamoeba* sp. also affect the lungs, liver, kidneys, adrenal glands, heart, skin and bones [7,9,85]. These are rare infections, mainly occurring in immunosuppressed patients, including those after organ transplantation [25].

Cutaneous acanthamoebosis is characterized by spots and single or multiple nodules which can increase size and become ulcerated. Skin lesions mainly affect the face, trunk and limbs. The risk factors include postoperative scars, changes caused by chickenpox infection, bites and mechanical injuries [86]. Cutaneous acanthamoebosis, due to a similar clinical picture, is often mistaken for other skin diseases caused by bacteria, viruses, fungi or post-traumatic inflammatory changes. Frequent erroneous diagnosis of the disease results in mortality of approximately 70% [60,87]. Diagnosis is based primarily on the histopathological examination of the skin section, which contains granulocytes surrounding lymphocytes, giant cells, plasma cells, cutaneous and subcutaneous necrosis and neutrophil infiltrates [60]. In addition, immunofluorescence, breeding and PCR methods are used [27]. The treatment involves administration of chlorhexidine glucuronide and ketoconazole in combination with one of the following drugs: pentamidine isetate, sulfadiazine, flucytosine, fluconazole and thoraconazole [88]. Most of the cases of cutaneous acanthamoebosis reported to date concerned immunocompromised patients, but cases in immunocompetent patients have also been described [89].

Amoeba infections of the lung, liver and bone usually occur in patients with lower immunity levels. In patients with pulmonary acanthamoebosis, weight loss and decreased respiratory efficiency were observed while radiological examination revealed interstitial lesions with visible pulmonary oedema [90]. It was noticed that amoeba infection into the lungs can be bilateral with inconsistent infiltrations and is frequently accompanied by an already existing disease [91]. Diagnosis of the infection is usually performed *post-mortem* [8,85, 91,92].

Histopathological changes in the lungs, liver and kidneys of mice infected with *Acanthamoeba* sp. were described by Górnik and Kuźna-Grygiel [64]. In the lungs of the mice they found hyperplasia of the bronchiolar epithelium, thickening and congestion of the alveolar walls and trophozoites visible in the vascular walls. The authors observed extensive necrotic changes in the liver, accompanied by inflammatory infiltrates, polymorphism of hepatocyte nuclei and petechia, as well as extensive necrotic changes of the tubules and glomeruli and petechia in the kidneys [64].

In summary, *Acanthamoeba* sp. are cosmopolitan protozoa which pose a threat to the health and life of humans and animals due to their ability of development both inside and outside the host. As a result of their ubiquity, the widespread use of contact lenses and an increased number of persons with lower immunity levels, the incidence of *Acanthamoeba* sp. infection is likely to increase. More studies on the immunological and biochemical mechanism involved in the elimination of the parasite are necessary in order to fully elucidate the pathogenesis of diseases caused by the protozoa and to develop appropriate diagnostic and therapeutic strategies.

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