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Vannella sp. harboring Microsporidia-like organisms isolated from the contact lens and inflamed eye of a female keratitis patient

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Abstract Viable *Hartmannella* sp. and two strains of *Vannella* sp. – but no *Acanthamoebae* – multiplied on NN-agar inoculated with pieces of the contact lens from a female keratitis patient. Within the cytoplasm of one *Vannella* isolate, intracellular parasites could be observed whose earliest stages were developing within the nucleus, resembling those Microsporidia-like parasites seen within *Vannella* isolated recently from a warm tapwater system. This assumption was also confirmed by electron microscopy. In swabs taken directly from the cornea, *Pseudomonas aeruginosa* were identified, but they did not yield any growth of amebas in culture. However, cocultivation of parasite-free *Vannella* strains with the above-mentioned swab matter resulted in infected amebas harboring the same intracellular parasites seen before. This infection could be established only if the corresponding spores were present as infective agents in the swab matter. The successful treatment of the patient with antibiotics supports the assumption that *P. aeruginosa* was the main cause of the corneal ulceration. The extent to which the Microsporidia-like organisms may have been involved in the development of keratitis remains a matter of discussion.

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Introduction

Since Rowbotham (1980) first observed the intracellular multiplication of *Legionella pneumophila* – which causes legionnaires' disease – within phagosomes of *Acanthamoebae*, several other potentially pathogenic bacteria have been described as multiplying within *Acanthamoebae* and other free-living amebas (FLA). Among those endocytobionts were pathogenic species such as *Listeria* (Ly and Müller 1990), *Ralstonia pickettii* (Michel and Hauröder 1997), and *Pseudomonas aeruginosa* (Michel et al. 1995) as well as hitherto unknown species such as *Chlamydia*-like and *Ehrlichia*-like coccoid bacteria, the first of which were recently described as an acknowledged new species called *Parachlamydia acanthamoebae* (Everett et al. 1999). Thus, the distribution of the endocytoparasites is promoted by the corresponding host amebas, which also protect them to a certain extent against biocides such as chlorine.

Not only have prokaryotes been observed multiplying within FLA, but also eukaryotes such as microsporidian organisms have been detected within *Vannella* (Hoffmann et al. 1998), as have yeast-like organisms within *Thekamoeba similis* (Michel 1997). Those *Vannellae* harboring microsporidian parasites were isolated from a domestic hot-water supply. The sporogenic plasma of the endoparasites started to develop within the karyoplasm and differentiated after proliferative growth into characteristic spores, which were liberated into the environment on the rupture of the host ameba.

At the time of the aforementioned observation it was not clear whether these findings might be of any significance for consumers of potable water. However, similar, if not identical, Microsporidia-like parasites have very recently been observed in relation to a case of keratitis initially supposed to have been caused by

Acanthamoebae. A detailed description of the isolation of these particular endocytobionts is given below with a discussion of the question as to which consequences these unusual findings might have for the patient. These observations and considerations are of special interest since intraocular infections with different microsporidian species have been described elsewhere (Ashton and Wirasinha 1973; Canning and Lom 1986; Bryan et al. 1990; Shadduck et al. 1990; Cali et al. 1991).

Materials and methods

Case report

A 24-year-old woman presented to the emergency room of the eye clinic with a 4-week history of pain, photophobia, and other symptoms of inflammation in her left eye. Her visual acuity was reduced to the recognition of hand movements, and the cornea showed a central disciform, grayish-white infiltrate (Fig. 1a). The conjunctiva was hyperemic, and the anterior segment showed only a few cells in the aqueous humor as a sign of inflammation. Given the patient's long-standing history of contact lens use, we took corneal swabs for culture as well as antibiotic-sensitivity testing to rule out *Acanthamoeba* infection. In addition, the contact lenses and their storage case were sent for further evaluation to the Institute of Parasitology in Bonn.

After a working diagnosis of infectious corneal ulcer had been established, a broad range of local antibiotic treatment was started, with 0.9% gentamicin eye drops and 10% Cefalozin eye drops being applied every hour. To cover the possibility of *Acanthamoeba*-induced keratitis, Brolene eye drops were also applied every hour and ointment was used at night.

Within 2 days of the beginning of local therapy the infiltrate started to regress. The frequency of local drug application was gradually reduced. Over a period of 16 days the ulcer healed and the epithelial layer closed, leaving a central discoidal white scar. The local administration of Brolene eye drops was continued for several weeks. Finally, only local substitution with artificial tears was needed. The visual acuity of the left eye was reduced to 1/25 due to the central scar (Fig. 1b). However, to date the patient has refused to undergo a perforating keratoplasty for visual rehabilitation.

Parasitology studies

Swabs from both eyes of the patient and both contact lenses were transferred to water-agar seeded with *Escherichia coli* as nutrient bacteria and were then incubated for at least 8 days at 30 °C. The multiplying amebas observed on one plate were subcultured onto NN-agar as described by Page (1976, 1988). After pure cultures of the different ameba species had been isolated, they were identified by morphological features according to Page (1988). The spores of the observed intracellular parasites, obtained after the bursting of the heavily infected host amebas, were transferred to the parasite-free strain Dent G1, the host *Vannella* strain of KW19. The cultures were studied by phase-contrast light microscopy using a Leica Ortholux II device.

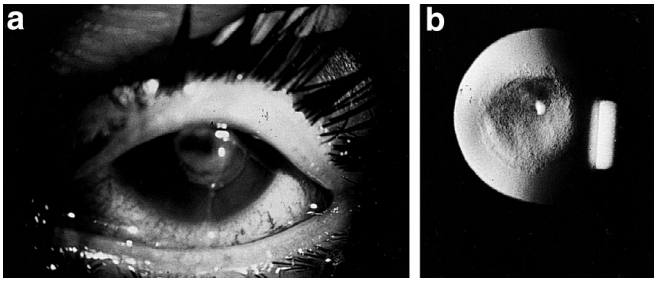


Fig. 1a, b Condition of the left eye of a patient with keratitis. **a** Condition as determined in the emergency room: corneal ulcer with a central discoidal infiltrate and conjunctival hyperemia. **b** Discoidal scar remaining after treatment (frontal light and slit-light illumination)

Electron microscopy studies

From the 3rd to the 5th day after infection the parasitized *Vannella* were harvested from NN-agar plates and then concentrated to a pellet by centrifugation at 1800 rpm for 10 min. The pellet was immediately fixed with 3% ice-cold glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.2). After prefixation the specimens were fixed in 1% osmium tetroxide and then in 2% uranyl acetate in aqueous solution. Subsequently, specimens were dehydrated in alcohol and embedded in Spurr's epoxy resin. Thin sections were stained with 1% lead citrate and examined using a Zeiss EM 10 electron microscope.

Results

The swabs taken from both eyes of the female patient yielded no growth of primary FLA. This matter was transferred to the surfaces of agar plates and subsequently used as described below.

The plates provided with pieces of the right contact lens also showed no growth of FLA. However, incubation of the plate containing matter from the left eye (afflicted with keratitis) resulted in the growth of different FLA. After pure subcultures of three strains had been obtained, one of these was identified as *Hartmannella vermiformis* and two, as *Vannella* species. The small strain was identified as *V. platypodia* and the medium-sized strain, as *Vannella* sp. The cellular membrane of the latter strain was not decorated by glycostyli (Figs. 3, 7b), which are characteristic of the genus *Vannella*, according to Page (1988). Since all other features such as the cell shape, the type of movement, and the floating form were absolutely characteristic of the genus, this strain was designated *Vannella* sp. As is generally known, *Hartmannella* form cysts, in contrast to *Vannella*.

Additionally, in one subculture of *Vannella*, single trophozoites exhibited large intracellular aggregates of parasites (Fig. 2a, b). These heavily infected cells underwent rupture within a period of 1–2 days and released considerable numbers of coccoid organisms into the environment of the agar surface as evidenced by phase-contrast microscopy. Since these parasitic aggregates resembled the same stages previously described as Microsporidia-like organisms (Hoffmann et al. 1998), we started to breed new subcultures from the few medium-sized infected *Vannella* to provide larger amounts of infected trophozoites for electron microscopy. The electron micrographs (Figs. 3–5) revealed the following features. In an overview (Fig. 3), intracellular sporogenic plasma and a single spore were observed. A group of free spores obtained from the agar surfaces

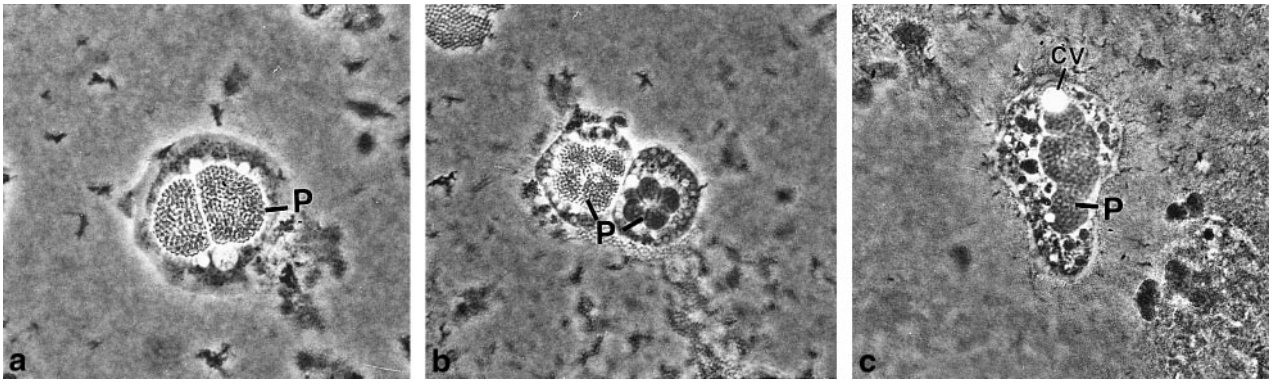
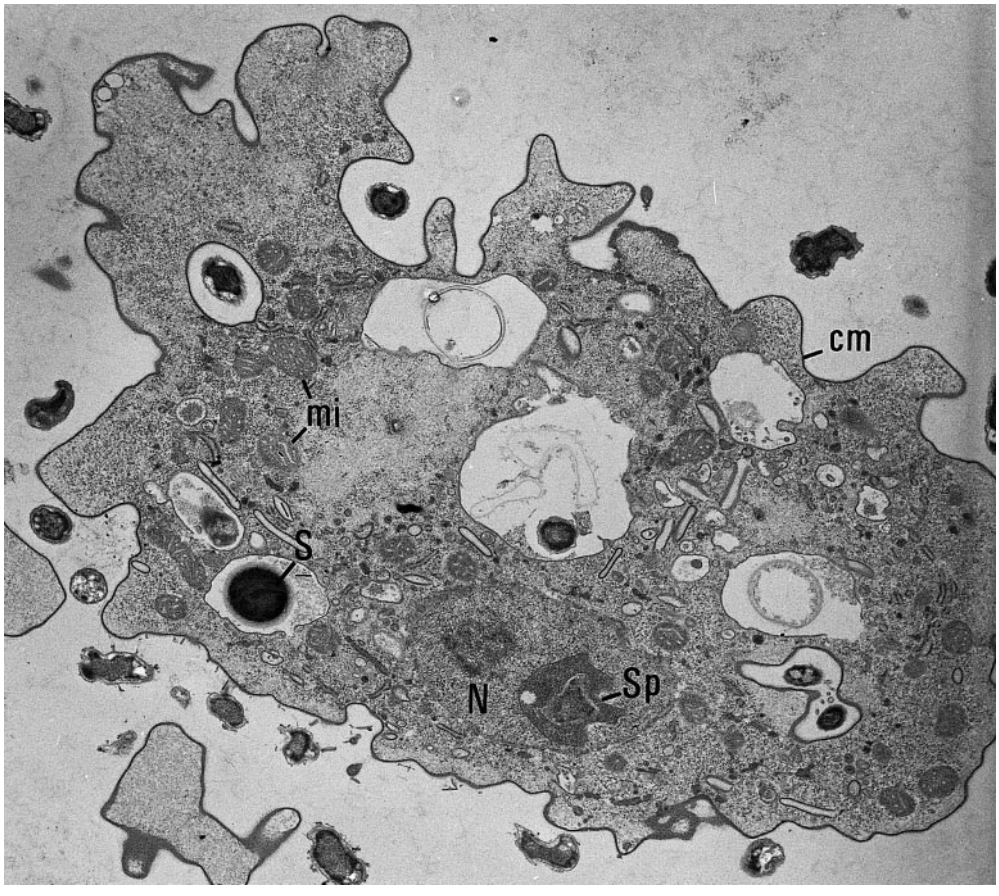


Fig. 2a–c *Vannella* sp. with intracellular parasites (*P*). **a** Sporogenic plasma of 2 parasites has differentiated into more or less mature spores. **b** Two *Vannella* harboring 4 and 5 parasites, respectively. The *right ameba* harbors immature sporogenic plasma and the *left ameba*, a parasite differentiating into spores. **c** *V. miroides* (strain DentG1) after the transfer of liberated spores to these other possible host amebas. The host remains active as evidenced by the contractile vacuole (*CV*) and the elongated shape, signs of active locomotion. ×400

(Fig. 4) exhibited a prominent nucleus, irregularly coiled polar filaments, vacuoles, and a distinct polaroplast as characteristic features of microsporidial spores. At higher magnification (Fig. 5) the nucleus and polaroplast could be seen very clearly. In addition, separation into the endo- and exospore could be recognized in the lower spore shown in Fig. 5. The endospore also showed sites of invagination.

Parallel to cultures with parasitized *Vannella*, the parasite-free strain of *Vannella* sp., strain Aun0, was kept for attempts at xenodiagnosis using the undefined matter obtained from both the keratitis-afflicted eye and the healthy eye. For this purpose, trophozoites from the advancing ring of trophozoites multiplying on the agar-plate surface were cocultivated with the bacteria containing matter for 4 days. After this period a few trophozoites on the agar plates containing matter from

Fig. 3 *Vannella* sp. displaying intranuclear sporogenic plasma (*Sp*) and a spore (*S*) within a vacuole (*N* Nucleus of the host ameba, *cm* cellular membrane, *mi* mitochondrion). ×8,000



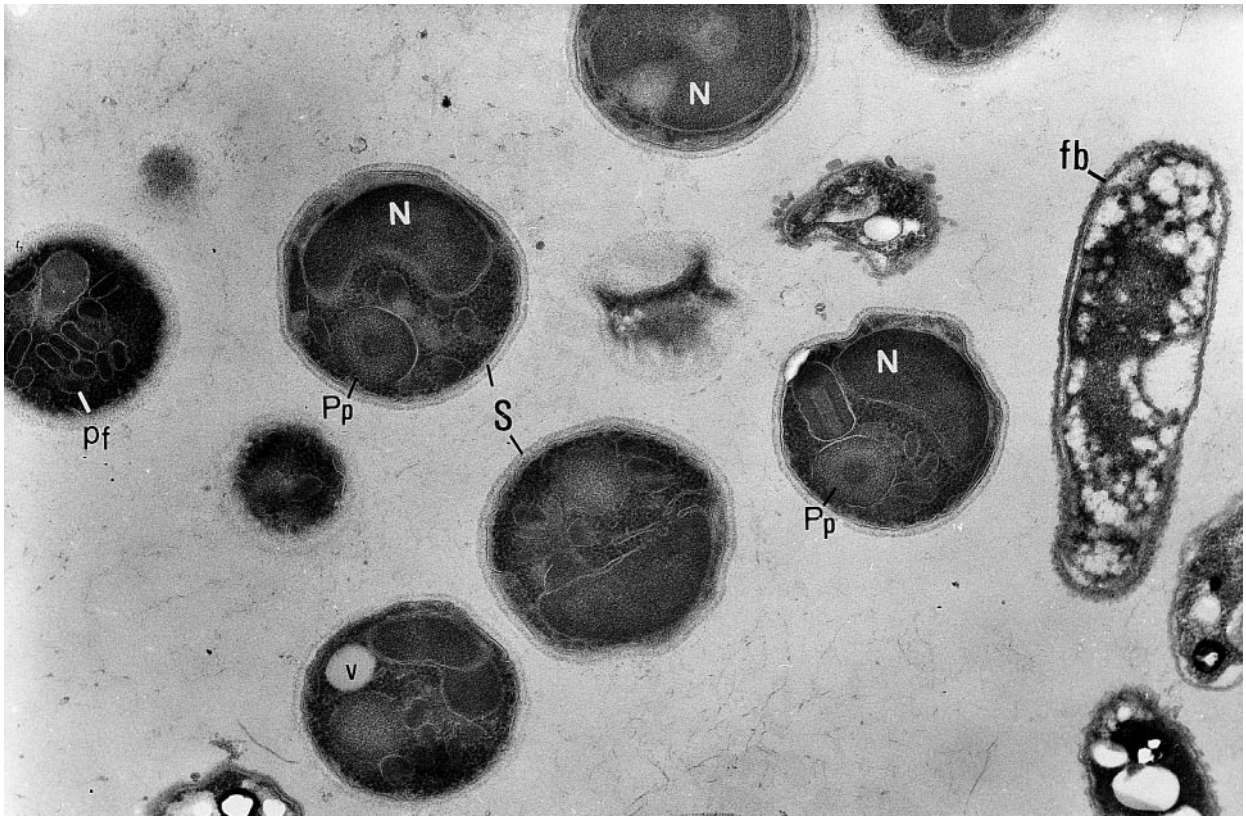


Fig. 4 A group of spores (*S*) after the rupture of the host ameba. They are characterized by a large nucleus (*N*), irregularly arranged polar filaments (*Pf*), and a vacuole (*v*); two spores exhibit polaroplasts (*Pp*). ×28,000

the left eye showed signs of infection (Fig. 2c) indistinguishable from the first findings observed within *Vannella* (Fig. 2a, b) from the left contact lens after cocultivation with strain Aun0. This phenomenon was not induced by the matter from the right eye.

Electron microscopy produced comparable results in these artificially infected amebas. The morphology of the parasites was the same as that of the infected amebas from the contact lens (Figs. 6, 7). Sporogenic plasma and spores within the same trophozoite were observed (Fig. 6). In this case, four to five ghosts of spores were detected in addition to two intact spores with prominent nuclei. It is not clear whether the ghosts might have been the result of digestion. Again, within one mature magnified spore, corresponding organelles such as the nucleus, the polar filaments, the polaroplast, the endospore, and the exospore were clearly discernible (Fig. 7a). Another obviously immature spore located within the cytoplasm of the host ameba showed a prominent nucleus and was packed with great numbers

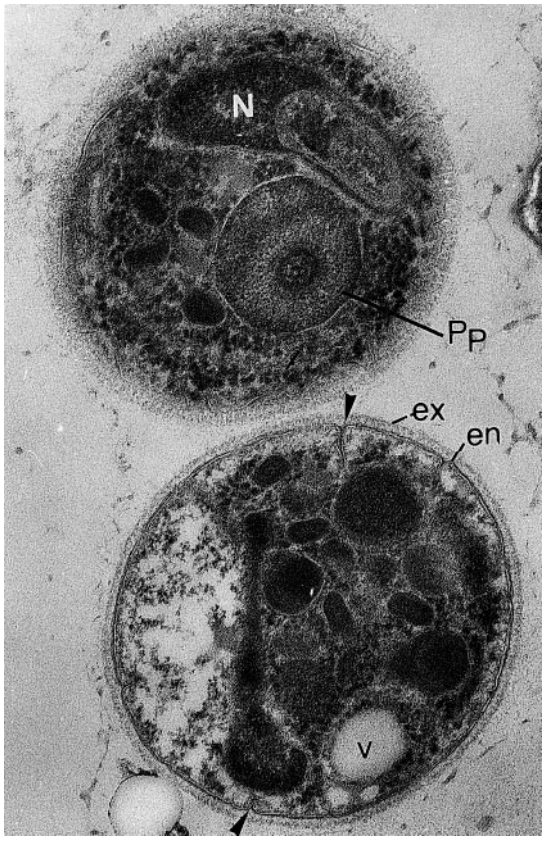


Fig. 5 Two spores of the endocytobiont AunI from *Vannella* are very clearly visible. The *upper spore* shows a nucleus (*N*) and a polaroplast (*Pp*), whereas separation into the endospore (*en*) and exospore (*ex*) and a distinct invagination (arrowheads) can be recognized in the *lower spore*. ×40,000

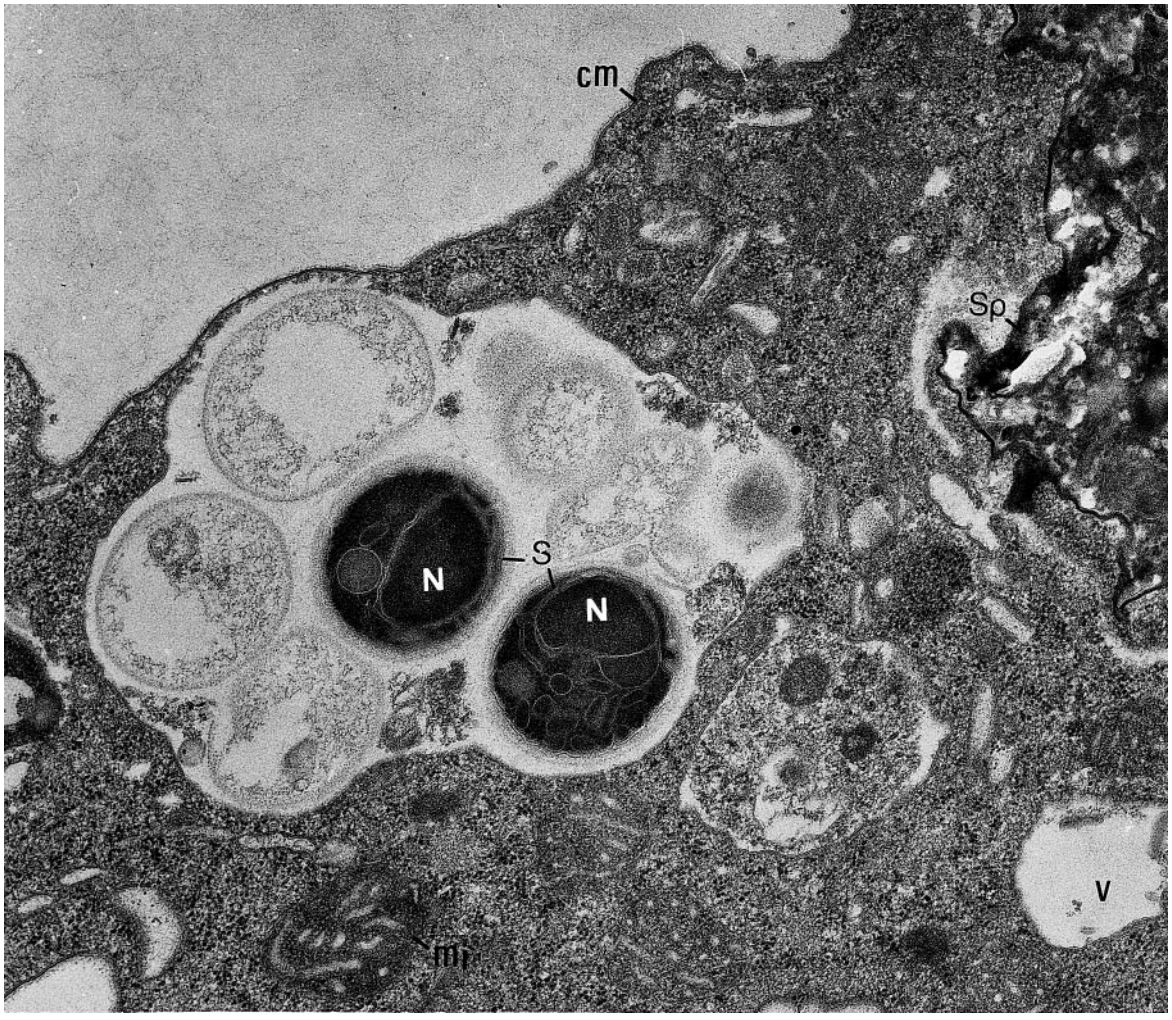


Fig. 6 *Vannella* sp. with the parasite AunII. The result of cocultivation of *Vannella* sp. with swab matter from the left eye was infection of the trophozoite with sporogenic plasma (*Sp*) and two intact spores (*S*) within a phagosome also containing the ghosts of 4–5 empty spores. Note that the cellular membrane (*cm*) of the ameba does not exhibit glycostyli (*N* Nucleus of the spore). ×26,000

of ribosomes of high electron density but did not contain any exospore (Fig. 7b). The cellular membrane of the host ameba, strain Aun0, was not decorated with glycostyli characteristic of the genus *Vannella*; instead, the ameba's glycocalyx was formed by a fine fuzzy coat.

These findings, obtained by the comparison of two batches of infected amebas that developed independently from each other, support the conclusion that the different stages found belong to the same Microsporidia-like parasites.

Discussion

It must be emphasized that in contrast to *Acanthamoebae*, neither *Hartmannella* nor *Vannella*, both of which were isolated from the contact lenses of our patient, are considered to be causative agents of keratitis, although Aitken et al. (1996) have observed trophozoites of *Hartmannella* and *Vahlkampfia* within the corneal stroma of a patient with keratitis. Apart from this consideration, the detection of three strains of FLA adhering to the left contact lens nevertheless indicates the poor hygienic condition of the contact-lens storage case.

In a comparison the Microsporidia-like organisms isolated from the contact lens and from the affected eye of the patient could not be distinguished from the endocytobionts only recently described for the first time as *Amoebosporidium minutum* (Hoffmann et al. 1998). Both intracellular parasites started developing within the karyoplasm of the nucleus, where they grew until they tore the nuclear membrane and differentiated into spores measuring 1–1.3 μm in diameter, which were released into the environment on the rupture of the host ameba. The liberated spores could then be ingested by hitherto uninfected amebas. In either case it is not yet clear how the content of the spores manages to penetrate into the nucleus to start the development of sporogenic plasma.

Remarkable was the evidence of spores in the affected eye as determined by xenodiagnosis using parasite-free *Vannella*. This occurrence of spores in the eye raises two

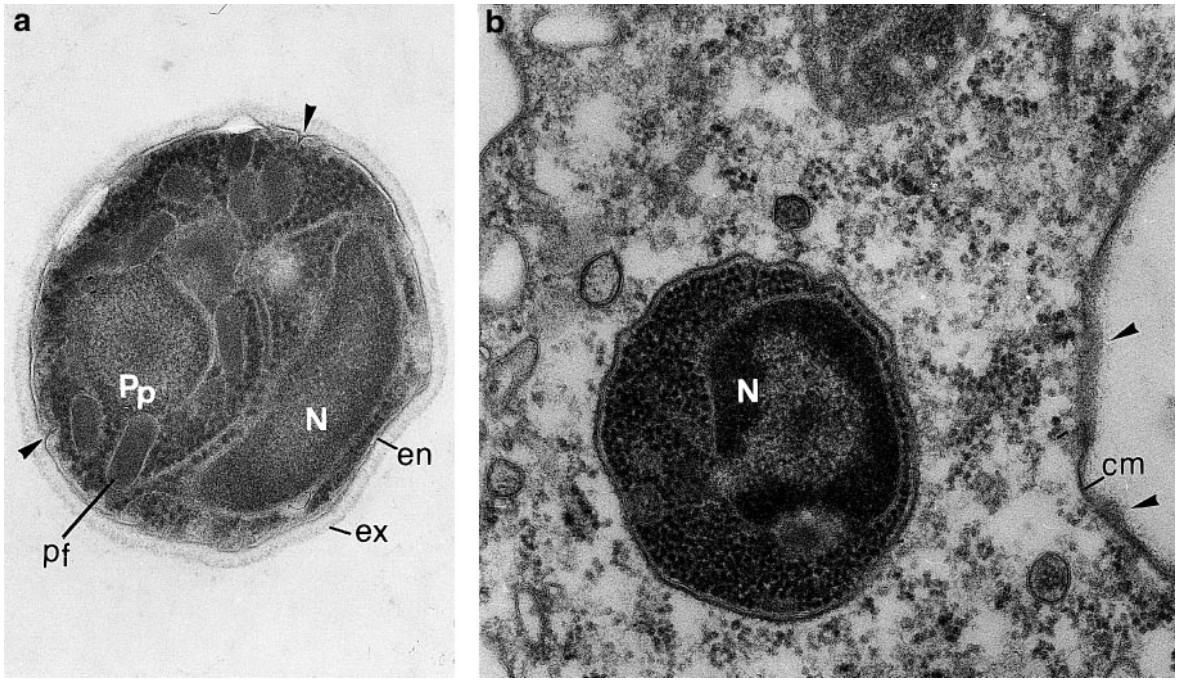


Fig. 7a, b Two spores of the Microsporidia-like parasite AunII after the differentiation of parasites. **a** Morphologically identical mature spores with nuclei, polar filaments, and polaroplasts (Pp) are enveloped by the endospore (*en*) and exospore (*ex*) and show invaginations at some points (*arrowheads*). × 50,000. **b** A spore with a prominent nucleus (*N*) but no exospore is tightly surrounded by the cytoplasm of the host *Vannella*. The cellular membrane does not display discernible glycostyli but shows a glycocalyx formed by a simple fuzzy coat (*arrowheads*). ×50,000

major questions: how did the parasite get into the affected eye, and what does this mean for the patient? First, the spores may have been passively transmitted to the eye by their host amebas. This would have no meaning for the affected eye. Second, according to the observation of Aitken et al. (1996), the host *Vannella* and/or *Hartmannella* could have invaded the cornea as a result of bacterial activity, since *Pseudomonas aeruginosa* was found and considered to be the main cause of this corneal ulceration. The third and final consideration is most unlikely: that the spores from burst *Vannella* themselves penetrate into epithelial cells by means of their polar filaments and start an independent cyclic form of development such as that which is well known for other microsporidians capable of causing keratitis, e.g., *Encephalitozoon hellem* or *E. cuniculi*. Information with respect to the latter two possibilities could be gained only from serial sections of the removed cornea. As stated above, the patient has not yet agreed to undergo the proposed keratoplasty.

Corneal stroma-infecting microsporidians were first described by Ashton and Wirasinha as early as in 1973. *Microsporidia* called *Microsporidium* (Canning and Lom 1986) and *Nosema*, e.g., *N. corneum*, from a patient with acquired immunodeficiency syndrome (AIDS) were subsequently described (Bryan et al. 1990; Shadduck et al. 1990). Later, all these different parasites were affiliated with only two species of *Encephalitozoon*, i.e., *E. hellem* and *E. cuniculi* (Cali et al. 1991). Their elongated spores measure 1 × 2 μm and are about twice as long as the spores of the parasites described above, which have an average length of only 1 μm and a rounder shape than *Encephalitozoon* spores.

Another important difference is the intranuclear growth and differentiation of the microsporidians described in this paper as compared with the development of the sporogenic plasma of *Encephalitozoon* within the cytoplasm of the host cell. Very few Microsporidia exhibit intranuclear development, e.g., *Enterocytozoon salmonis* from salmonid fish (Chilmonczyk et al. 1991) or a similar species from the Atlantic halibut (Nilsen et al. 1995). However, apart from this mode of development, the spores of these species differ in size and shape; they have a slender shape and measure 2.9 μm in length.

Thus, the tiny Microsporidia from *Vannella* are not comparable with any known microsporidian species. For the future it would be thrilling to find out whether the amebic parasites might also be isolated from another animal or human host. That undoubtedly identical endocytobionts of differing origin were recognized within such a short period indicates that they may not be as rare as the late detection of these unique parasites might suggest.

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