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SHORT COMMUNICATION

Early diagnosis of amoebic keratitis due to a mixed infection with *Acanthamoeba* and *Hartmannella*

Jacob Lorenzo-Morales · Enrique Martínez-Carretero · Ninive Batista · Jorge Álvarez-Marín · Yasmina Bahaya · Julia Walochnik · Basilio Valladares

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Abstract A mixed keratitis due to *Acanthamoeba* and *Hartmannella* species is reported. The patient was a soft contact lens wearer. Early diagnosis was achieved by polymerase chain reaction and culture. The pathogenic potential of the isolated amoebae was proven using cytotoxicity assays. The reported case underlines the difficulties in identifying a corneal amoebic infection. In our case, the early diagnosis of a mixed infection allowed a proper antiamoebic treatment in an early stage of infection. This may have been the reason of a successful outcome after therapy.

Free-living amoebae of the genera *Acanthamoeba* and *Hartmannella* are ubiquitous in nature. *Acanthamoeba* spp. are well known as causative agents of severe eye infections, occurring mainly in immunocompetent contact lens wearers. However, also other species such as *Hartmannella* and *Vahlkampfia* have been occasionally reported to be associated with keratitis (Aitken et al. 1996; Barker and Brown 1994; Mah-Sadorra et al. 2005). To our knowledge, this is the third report of corneal coinfection due to *Hartmannella* and *Acanthamoeba* species. In previous reports (De Jonckheere and Brown 1998, 1999), no establishment or quantification of the pathogenic potential of the isolates was determined. Although *Hartmannella* has been already included in the list of human parasites (Weekers et al. 1994), its role in the development of human pathologies remains controversial (De Jonckheere and Brown 1998, 1999). However, the pathogenecity assays which were undertaken in this study as well as the ability of *Hartmannella* to grow at high temperatures are supporting the idea of this protist being a rare causative agent of human keratitis. Moreover, amoebae of the genus *Hartmannella* are of substantial medical relevance as potential vehicles for pathogenic bacteria. They are well known to play a role vectors for *Legionella pneumophila* (Fields et al. 1990) and have also been shown to be suitable hosts for pseudomonads, which are considered to be the most important eye pathogens in contact lens wearers (Mah-Sadorra et al. 2005).

A 21-year-old man was admitted to the Department of Ophthalmology, Hospital Universitario Nuestra Señora de La Candelaria, Tenerife, Spain, with a 2-week history of severe pain, photophobia, and impaired vision. On examination, he presented a stromal keratitis with satellite focuses and cilliary injection in the right eye. It is also important to mention that the patient was a soft contact lens wearer who did not maintain standard contact lens care. Superficial and deep scrapes were taken from the cornea to examine possible involvement of any bacterial, fungal, viral or parasitic pathogens. No pathogenic bacterial, viral, or

J. Lorenzo-Morales (✉) · E. Martínez-Carretero · B. Valladares
University Institute of Tropical Diseases and Public Health
of the Canary Islands, University of La Laguna,
Avda. Astrofísico Fco. Sánchez, S/N,
38203 La Laguna, Tenerife, Canary Islands, Spain
e-mail: jmlorenz@ull.es

N. Batista
Microbiology Unit, Hospital Nuestra Señora de La Candelaria,
Ctra. Del Rosario, S/N,
38010 Santa Cruz de Tenerife, Canary Islands, Spain

J. Álvarez-Marín · Y. Bahaya
Department of Ophthalmology,
Hospital Nuestra Señora de La Candelaria,
Ctra. Del Rosario, S/N,
38010 Santa Cruz de Tenerife, Canary Islands, Spain

J. Walochnik
Department of Medical Parasitology,
Medical University of Vienna,
Vienna, Austria

fungal agents were found. However, *Acanthamoeba* and *Hartmannella* trophozoites were recovered from both scrapes, after cultivation on 2% non-nutrient agar plates covered with a layer of heat-inactivated *Escherichia coli*. Contact lenses and contact lens cases were also positive for the presence of both, *Acanthamoeba* and *Hartmannella* species. Genus-specific polymerase chain reactions (PCRs) for *Acanthamoeba* and *Hartmannella*, respectively, were carried out directly from the corneal scrapes, contact lenses, and their cases to avoid possible contaminations. Amplification of specific PCR products proved the presence of both genera in all studied samples. The patient was treated with Tobramicin (Tobradex®), propamidine isothionate (Brolene®), and povidone iodine (Betadine®), five times per day until the corneal lesion healed. After that, diclophenac and ofloxacin (Exocin®), each three times per day, were also administered for 6 weeks until the patient had recovered. After a week of treatment, the patient showed a good response to the therapy with cicatrisation of the cornea. Six weeks after initiation of the therapy, the patient's status was favorable, and he recovered his visual acuity up to 95%.

The isolated amoebae were cloned by dilution from the original agar cultures to obtain axenic cultures for cytotoxicity assays of each amoeba, separately. The presence of monoaxenic cultures was confirmed by microscopy and genus-specific PCR. *Acanthamoeba*-specific PCR was carried out using genus-specific primers as previously described (Vodkin et al. 1992). For each amplification, two different type strains of *Acanthamoeba* from the America Type Culture Collection (ATCC; ATCC 50238 and ATCC 50492, both genotypes T4) were used as positive controls. Furthermore, the genotype of the *Acanthamoeba* isolate was determined as T4 using the previously described methodology (Booton et al. 2005). *Hartmannella* PCR was carried out using an 18S rDNA specific primer pair, and the reactions were undertaken as previously described (Inoue et al. 1998). For each amplification, a type strain of *Hartmannella vermiformis* from the Culture Collection of Algae and Protozoa (CCAP 1534/7A) was used as a positive control. The obtained amplicon was purified using the Qiaquick PCR Purification kit (Qiagen, Hilden, Germany) and sequenced using an ABI 310 automated fluorescent sequencing system (Applied Biosystems, Foster City, CA, USA).

The obtained sequence of *Hartmannella* spp. showed an unusual low identity (between 97.4 and 98.23%) to the other published *Hartmannella* sequences in the GenBank database. The nucleotide sequence data from both isolates were deposited at the GenBank and are available under the following accession numbers: E205324–E205325.

Furthermore, the pathogenic potential of both isolates was established using cytotoxicity assays, determining the release of lactate from HCE-2 epithelial corneal cells (ATCC # CRL-11135, LGC Promochem, Barcelona, Spain), as previously described (Lorenzo-Morales et al. 2005). Briefly, the amoebae were added to the epithelial cells in 24-well plates, and cultures were incubated in a 5% CO_2 incubator at 37°C for 12–24 h. After this incubation, cytotoxicity was determined. Epithelial cells without parasites were used as controls. The experiments were repeated three times with similar results for each amoebae strain. The obtained results showed that both amoebae were able to produce cytotoxicity effects on this particular cell line. However, the cytotoxicity levels for the *Acanthamoeba* isolate were higher (87.6%) compared to the effects which were produced by the *Hartmannella* isolate (64.3%) for the established period of time. These results are consistent with those previously described (Kinnear 2003).

The *Acanthamoeba* isolate was identified as genotype T4, which is the main genotype being involved in amoebic keratitis infections worldwide (Khan 2006). As expected, the cytotoxicity assays carried out showed that this amoeba exhibits a high pathogenic potential against corneal epithelial cells. However, it remains a question which one out of these two isolates is responsible for the pathology. In previously reported cases of amoebic keratitis coinfections (De Jonckheere and Brown 1998, 1999), poor response to the therapy was described. These authors were discussing either resistance of *Hartmannella* to antiamoebic treatment or bad penetration of drugs into the deeper layers of the cornea, the site that *Hartmannella* has been isolated from in these cases. In case of the former, the possibility of a coinfection should be investigated in cases of amoebic keratitis with a poor response to the therapy. Unfortunately, there are no available drug sensitivity studies on *Hartmannella* spp.; thus, further studies are necessary.

In contrast to these previous data, our patient's response to therapy was favourable. However, in our case, the diagnosis was established in an early stage of the infection, and thus, the used antiamoebic treatment might have been successful. In the previous reports, unfavorable outcome could be also due to late identification of the causative agents, which were misdiagnosed as herpes simplex.

The reported case underlines the difficulties in identifying a corneal amoebic infection. In our case, the early diagnosis of a mixed infection allowed a proper antiamoebic treatment in an early stage of infection. This may have been the reason of a successful outcome after therapy.

In conclusion, an early suspicion of amoebic keratitis is important as an early diagnosis might lead to specific early treatment with good response to the therapy.

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