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SHORT COMMUNICATION

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Isolation and identification by partial sequencing of the 18S ribosomal gene of free-living amoebae from necrotic tissue of *Basiliscus plumifrons* (Sauria: Iguanidae)

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Abstract A 3-year-old *Basiliscus plumifrons* developed a necrotic lesion on the tail resulting from nodules of unknown etiology. Investigation of necrotic tissue revealed several gram-negative bacteria as well as three different species of free-living amoebae. The amoebae were identified by morphological characters as belonging to the genera *Acanthamoeba*, *Echinamoeba*, and *Naegleria*, respectively. Partial sequencing of the 18S ribosomal gene was performed for reliable systematic determination. Two of the isolates showed thermotolerance. No isolate was growable in conventional liquid media, but the *Acanthamoeba* strain readily grew on a human cell line (HEp2). It remains unclear whether the amoebae fed on the coexisting bacteria or on host tissue.

Introduction

Free-living amoebae are widely distributed in natural and artificial habitats. Apart from their occurrence in fresh water, seawater, and soil, they have been found in several animals as well as in Amphibia (Frank and Bosch 1972; Madrigal Sesma et al. 1988) and Reptilia (Bosch and Deichsel 1972; Frank and Bosch 1972; Madrigal Sesma et al. 1988).

Although free-living amoebae have repeatedly been isolated from asymptomatic individuals, e.g., from human nasal mucosa (Cerva et al. 1973; Ockert 1974; Michel et al. 1982), the ability of these protozoa to cause fatal disease in humans is well established (Ma et al. 1990). Several studies have also identified free-living amoebae as causative agents of diseases in animals. Amoebic meningoencephalitis in mammals has been described in several reports, e.g., in an orangutan (Canfield et al. 1997) or in a South American tapir (Lozano-Alarcon et al. 1997). Bosch and Deichsel (1972) have isolated amoebae of the "*Acanthamoeba-Hartmannella*" group from reptiles with diagnosed "brain amebiasis."

In the present study, three different free-living amoebae were isolated from necrotic tissue of a *Basiliscus plumifrons*. The isolates were characterized by morphological and molecular biological means.

Materials and methods

A 3-year-old female *Basiliscus plumifrons*, which had an amputation of the distal end of the tail with good recovery several months previously, again developed a necrotic lesion on the tail resulting from nodules of unknown etiology. Within the following 10 days, approximately 15 cm of the distal part of the tail became necrotic, which led to the loss of a 3.5-cm piece of tail. This piece was brought to the laboratory after 24 h, where it was disinfected externally with 70% ethanol. Necrotic material from the interior was obtained for bacteriological and parasitological investigation. Gram and Ziehl-Neelsen stains were performed for detection of bacteria. The bacteria were cultivated on Columbia agar and were identified by their analytical profile indices (API 20E, API 20 NE; bioMérieux, Marcy-l'Etoile, France).

Surprisingly, the native sample revealed trophozoites of different free-living amoebae. The amoebae were cultured on NN (nonnutritive)-agar plates coated with 100 µl of a suspension of heat-killed *Escherichia coli*. As the amoebae would not initially grow because of heavy bacterial contamination, the suspensions were supplemented with 200 IU penicillin/ml and with 200 µg streptomycin/ml to kill the concurrent bacteria. The isolates were cloned by transfer of a single cyst onto a fresh plate using a mic-

The nucleotide sequence data reported in this paper are available in the EMBL, GenBank, and DDJB data bases under the accession numbers AF114438 and AF114439

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romanipulator. Temperature tolerance (37° and 42 °C) and cell-culture pathogenicity (HEp 2) were determined. The enflagellation test was performed on the *Naegleria* isolate by mounting of the plate culture with 0.9% saline and observation of the transformation to the flagellate stage after 5–10 min.

Whole-cell DNA of the isolates was extracted by a modified UNSET (Hugo et al. 1992) procedure. In brief, amoebae were brought to extensive growth and amoeba trophozoites were harvested from the plates with sterile cotton-tipped applicators ($\sim 1 \times 10^5$ amoebae), washed three times in amoeba saline (Page 1991), pelleted by centrifugation (1,000 *g*/8 min), and resuspended in 500 µl of UNSET lysis buffer [8 *M* urea, 0.15 *M* NaCl, 2% sodium dodecyl sulfate, 0.001 *M* ethylenediaminetetraacetic acid (EDTA), 0.1 *M* TRIS-HCl, pH 7.5]. An equal amount of PCI (phenol-chloroform-isoamyl alcohol) was added immediately, and the solution was shaken gently for 5 h. The suspension was centrifuged at 3,000 *g* for 10 min, and the upper, aqueous phase was transferred to a new tube. PCI extraction (10 min) was repeated two times. Nucleic acids were precipitated by ethanol (15 min at −70 °C), pelleted at 12,000 *g* for 30 min at 4 °C, and resuspended in 25 µl of distilled water. We used 0.8 µl of whole-cell DNA and a standard amplification program (30 cycles; 1 min, 94 °C, 2 min, 50 °C; 3 min, 72 °C) polymerase chain reaction for (PCR) amplification.

Partial sequencing for rapid determination

Approximately 1,700 bp of the 18S ribosomal genes were amplified using an internal primer (5′-GGTAATTCCAGCTCCAATAG-3′) and the SSU2 primer (Gast et al. 1994) complementary to the 3′ end of the gene, both annealing to the genes of all three genera. Direct sequencing of PCR products was performed with API PRISM (Perkin-Elmer, Langen, Germany). Partial sequences from the 18S ribosomal gene were aligned to published sequences for systematic determination.

Sequencing for accurate identification

The whole gene of the *Acanthamoeba* isolate was amplified using the SSU1 and SSU2 primers (Gast et al. 1994) and then sequenced by direct sequencing. For the *Naegleria* isolate the ~800 bp used to study *Naegleria* spp. (De Jonckheere 1994) were amplified and sequenced directly from the PCR product.

Results and discussion

The bacteriological investigations revealed three bacterial species, namely, *Serratia marcescens*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*. Atypical mycobacteria were not detected. Three different amoeba strains were isolated, which were identified as belonging to the genera *Acanthamoeba*, *Echinamoeba*, and *Naegleria*, respectively (see Fig. 1), by morphological and physiological means according to Page (1991). The genus *Acanthamoeba* has been divided into three morphological species-groups (*Acanthamoeba* sp. Gr. I–III) by Pussard and Pons (1977). Our isolate belongs to *Acanthamoeba* sp. Gr. II. Recently, Stothard et al. (1998) identified 12 *Acanthamoeba* 18S rDNA sequence types. By partial sequencing (~300 bp) of the 18S rDNA and comparison with defined amoeba isolates the *Acanthamoeba* was identified as an *Acanthamoeba* of the sequence type T4. Interestingly, the vast majority of keratitis-causing *Acanthamoeba* strains seem to belong to sequence type

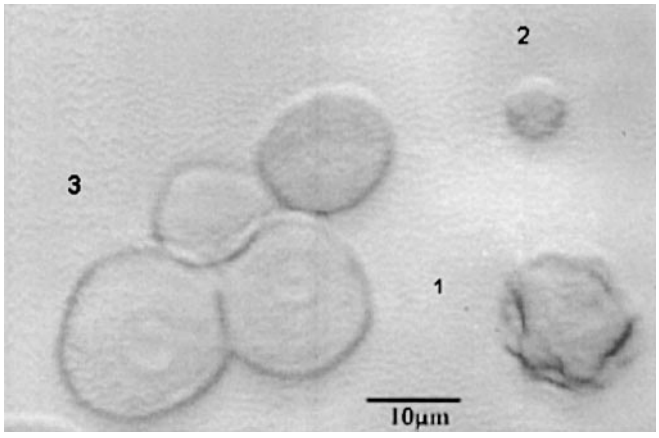

Fig. 1 Cysts of *Acanthamoeba castellanii* (*1*), *Echinamoeba* sp. (*2*) and *Naegleria* sp. (*3*) isolated from a basilise

T4 (Stothard et al. 1998). The partial sequencing of the *Naegleria* SSU ribosomal gene revealed highest identity to a *N. gruberi* (GenBank Ref. No. M18732) for our isolate. For the third isolate, which had been identified as *Echinamoeba* by morphological characters, the highest identity (~90%) was found to a *Hartmannella vermiformis* strain (GenBank Ref. No. M95168). As no *Echinamoeba* sequence was found to have been published and the trophozoites of our isolate showed typical Echinopodida, which do not occur in the Hartmannellidae, we define the isolate as *Echinamoeba* sp.

The described internal primer anneals to the SSU ribosomal genes of all three investigated genera in spite of the phylogenetic distance. Nevertheless, it allows a rather accurate identification employing a single sequencing reaction, as the first 300 bp after the primer encode a variable site of reasonable specificity. Therefore, we propose that this primer could be useful for rapid identification. In our opinion, isolation of DNA directly from the plate culture, without the placement of amoebae into liquid culture, followed by direct sequencing of the PCR product with the use of only a single pair of primers for various isolates is a fast and very simple method and would also be useful for rapid determination of clinical isolates. Moreover, by DNA isolation from the plate culture, sequences of strains that do not grow in artificial liquid media can be obtained.

To ensure a more precise identification of the *Acanthamoeba* and the *Naegleria*, the whole gene was sequenced for *Acanthamoeba* and for *Naegleria* the 800 bp used to study this genus (De Jonckheere 1994) were sequenced. The *Acanthamoeba* isolate exhibits 99% sequence similarity to an *A. castellanii* (GenBank Ref. No. U07416) isolated from soil in California. As the type strain for this species (*A. castellanii* Castellani) also matched our sequence with 98% identity, we identify our isolate as belonging to the species *A. castellanii*. The *Naegleria* isolate could not be classified, as equal similarity was found to a *N. australiensis* (GenBank Ref. No. U80058), to a *Naegleria* sp. (GenBank Ref. No. Y10184), and to a

N. gruberi (GenBank Ref. No. M18732) with 95% identity. *N. gruberi* is the species most closely related to *N. australiensis* (Page 1991) – which is mainly distinguished by its lack of thermotolerance. As our isolate shows no thermotolerance, it is very likely that it belongs to *N. gruberi*. Nevertheless, a preference for low temperatures for optimal growth could also be an adaptation to the reptile host.

The amoebae were in the trophozoite stage when isolated. It remains unclear whether they fed on host tissue or on the syntopically occurring bacteria. It is well known that *P. vulgaris* and *S. marcescens* as well as *P. aeruginosa* can serve as nutrient sources for free-living amoebae. The *Acanthamoeba* isolate seems to depend on living bacteria for multiplication – extensive growth was achieved only by the provision of a 72-h-old culture of living *Escherichia coli* – whereas the other two isolates showed extensive growth even on heat-killed bacteria. None of the isolates was growable in conventional liquid media. Except for the *Naegleria* isolate, which showed growth neither at 37 °C nor at 42 °C, the amoebae were thermotolerant. Interestingly, the *Acanthamoeba* isolate could grow at higher temperatures only if living bacteria (*E. coli*) were offered as food organisms. Obviously, living bacteria are needed to induce excystment and multiplication in this case. In contrast to the other two isolates, the *Acanthamoeba* readily grew on a human cell line (HEp 2), resulting in complete lysis of the cells within 24 h, but only at 30 °C, not at 37 °C. Although thermotolerance is certainly not a prerequisite for pathogenicity in cold-blooded animals, it definitively cannot be assumed that the free-living amoebae had caused the disease in this case.

Nevertheless, several studies have shown that free-living amoebae can cause disease in cold-blooded animals, including all major groups of the Reptilia (Bosch and Deichsel 1972; Frank and Bosch 1972). To our knowledge, this is the first description of an isolation of free-living amoebae from a basilisc.

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