Filamoeba sp. Isolated from Hot-water Piping System, a Host of Legionella Closely Related to Legionella micdadei

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Summary. An amoeba was isolated from 56°C hot water, sampled in a piping system that was monitored for the presence of legionellae. The amoeba strain JIH56 was derived from the primary isolate by subculturing at 38°C. It was assigned to the genus Filamoeba Page, 1967 using light microscopical, ultrastructural and molecular criteria. In the cytoplasm of Filamoeba trophozoites were found Legionella-like bacteria. These were determined using Legionella-specific PCR assay followed by sequencing. Phylogenetic analysis and a similarity search revealed a close relationship of the newly determined legionella sequence with sequences of Legionella micdadei (syn. Tatlockia micdadei), the Pittsburgh pneumonia agent. Phylogenetic analysis of the Filamoeba sequences available to date suggests that the ATCC 50430 strain was misidentified.

Key words: Filamoeba sp., morphology, phylogeny, intracellular Legionella sp., phylogeny

INTRODUCTION

The fact that free-living amoebae serve as host cells for intracellular replication of Legionella spp. has been reported in a series of studies (Rowbotham 1980, 1986; Harf and Monteil 1988; Abu Kwaik et al. 1998a, Newsome et al. 1998; Fields et al. 1990, 2002), discussed in reviews on prokaryote-protists associations (Barker and Brown 1994; Horn and Wagner 2004) and analysed in a review on co-evolution of legionellae with fresh-water protozoa (Albert-Weissenberger 2007). Parallel isolations of Legionella spp. and free-living amoebae of the genera Acanthamoeba, Hartmannella, Naegleria, Vahlkampfia, Willaertia, Echinamoeba and Cichlidium that have been reported from diverse freshwater habitats, and especially from man made water systems indicate close association of both organisms (Henke and Seidel 1986, Paszko-Kolva et al. 1991, Rohr et al. 1998, Declerck et al. 2007, Hsu et al. 2009).

In vitro experiments evidenced that legionellae multiply within and are pathogenic for Acanthamoeba castellanii (Moffat and Tompkins 1992, Hacker et al. 1993, Helbig et al. 1993), A. palestinensis (Anand et al. 1983), A. polyphaga (Barker et al. 1992), Hartmannella vermiformis (Breiman et al. 1990, Fields et al. 1990,
Wadowsky 1991, Abu Kwaik 1996) and Naegleria sp. (Newsome et al. 1985). One strain of Willaertia magna was found to be particularly resistant to infection by Legionella pneumophila (Dey et al. 2009). Results of in vitro experiments improved detection methods of legionellae. Incubation of water samples containing amoebae was proposed as a step preceding isolation attempts on media supporting the growth of legionellae (Sanden et al. 1992). Co-culture with amoebae has been demonstrated to be an efficient tool also for detection of Legionella-like amoebal pathogens (LLAP) and other “amoeba resistant bacteria” (ARB) in human and environmental samples (Greub and Raoul 2004; Evstigneeva et al. 2009). Corroborating studies of amoebae naturally harbouring legionellae from environmental sources are not many, nevertheless they brought conclusive evidence about close relationships between Legio nella spp. and free-living amoebae (Harf and Monteil 1988, Newsome et al. 1998).

The aim of this study was to identify free-living amoeba that was isolated from hot water piping system positively tested for the presence of legionellae and clarify whether this amoeba isolate hosts legionellae.

MATERIALS AND METHODS

Water samples from three sampling sites (outlets) of hot-water piping system in a 50-year-old, five-storeyed house were regularly screened for the presence of Legionella spp., using standard culturing methods described elsewhere (ISO Documents). For isolation of amoebae, Petri dishes with non-nutrient (NN) agar were seeded with inactivated Enterobacter aerogenes and inoculated either with water samples directly or after their concentration by filtration or centrifugation. Agar plates were incubated simultaneously at 28°C or 38°C. The presence/absence of amoebae was tested daily for 10 days. An amoeba strain designated as JIH56 was isolated from the hot-water sample that was taken from the tap after 1 min flow of water, within which the temperature 56°C was recorded. This strain was maintained by regular subculturing for two month and then cysts were stored on agar plates in wet chamber at 4°C for almost three years. Thereafter, subcultures were prepared on non-seeded non-nutrient agar (at 38°C) for light microscopical, ultrastructural and molecular studies. Living amoebae were observed on agar plates (in situ) and in hanging drops prepared with amoeba saline (Page 1988). Olympus BX51 microscope equipped with Nomarski differential interference contrast optics and digital camera DP 70 was used for observation and documentation of amoebae. One-week-old agar plate cultures selected for examination by electron microscopy were fixed in situ with 3% glutaraldehyde in cacodylate buffer. Trophozoites and cysts were pelleted, postfixed in cacodylate-buffered 1% osmium tetroxide and embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and Reynold’s lead citrate, and examined in a JEOL JEM 1010 electron microscope.

DNA isolation, amplification and sequencing

DNA was extracted from the amoeba cell pellets using the JETQUICK Tissue DNA Spin Kit (Genomed, Germany) according to the manufacturer’s protocol. The PCR amplification of the full-length 18S rRNA gene from the Filamoeba strain was performed as described previously (Dyková et al. 2005). The sample of amoeba DNA (same as used for amoeba determination) and Legionella species-specific primers p1.2 (5’-AGGGTTGATAGGTAAAGAGC-3’) and cp3.2 (5’-CCACAGCTAGTTGACATCG-3’) were used to amplify a 386 bp product of a partial 16S rRNA gene according to Jonas et al. (1995). Except for primers, the other components of amplification mixture were the same as in the 18S rDNA amplification. The cycling conditions began with an initial incubation at 95°C for 5 min., followed by 40 cycles of denaturation at 94°C for 1 min., annealing at 56°C for 1.5 min. and extension at 72°C for 1 min. Finally, incomplete PCR products were extended for 10 min. at 72°C. The amplified products of both Filamoeba and Legionella SSU rDNA genes were gel-purified and cloned into pDrive Cloning Vector using the QIAGEN PCR Cloning Kit (Qiagen GmbH, Germany). Sequencing was carried out on an automatic sequencer ABI 3130x1 using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

Phylogenetic analyses of amoeba and legionella sequences

Both alignments prepared to analyze phylogenetic status of obtained sequences of Filamoeba sp. and Legionella sp., respectively, were created in the program ClustalX 2.0.6 (Larkin et al. 2007). They were trimmed and checked for ambiguously aligned positions in BioEdit (Hall 1999). The final length of alignments was 1802 bp (Filamoeba alignment) and 1437 bp (Legionella alignment). Note that our sequence of Filamoeba sp. contained only 387 bp, but we used complete SSU rDNA sequences for other legionellae (if available), to obtain better resolved tree. Both alignments were phylogenetically analyzed using three different methods. Maximum parsimony (MP) analyses and analyses using Fitch-Margoliash method with LogDet distances (LD) were run in PAUP* 4.0b10 (Swofford 2003). Heuristic searches were conducted with 100 repeated searches in which the starting tree was constructed by random taxa addition and swapped with the TBR algorithm. The number of bootstrap replicates was 1000 for both MP and LD, with only 10 repeated searches per replicate. Maximum likelihood analyses were provided by the program RAxML 7.0.3 (Stamatakis 2006) with the use of GTR + Τ model and rapid bootstrapping (1000 replicates). To compare similarity of Legionella sequences, sequence identity matrix was prepared in BioEdit (Hall 1999) from a dataset containing all Legionella sequences used in phylogenetic analyses, but the dataset was shortened so that all sequences contained only positions corresponding to the 387 bp of our new sequence.
RESULTS

Identification of amoeba strain

Repeated light microscopical examination of trophozoites and cysts revealed a conspicuous similarity of JIH56 strain with previously studied *Filamoeba* strains (Page 1967, Dyková et al. 2005). Of the two known *Filamoeba* species, morphology of trophozoites of JIH56 strain resembled more the type strain of *Filamoeba nolandii* than *F. sinensis* (Fig. 1). Details of ultrastructure were difficult to observe in trophozoites, the cytoplasm of which contained many vacuoles and bacteria in different stages of their degradation. However, the most important details, i.e., mitochondria with non-branching tubular cristae arranged in a parallel way, type of nucleus and very thin cell surface layer, supported assignment of the JIH56 strain to the genus *Filamoeba* Page, 1967. Taxonomic conclusion based on light microscopical and ultrastructural details was verified using the molecular marker. The SSU rDNA sequence determined for the strain under study (GenBank Acc No. GQ371176) was compared with the *Filamoeba* sequences available to date. Phylogenetic analysis of the sequences fully supported assignment of JIH56 strain to the genus *Filamoeba* and positioned the sequence of this strain to the clade containing sequences of the type strain of *F. nolandii* (UKNCC strain of freshwater origin) as well as the sequence of ATCC 50430 (American Type Culture Collection strain of marine origin) (Fig. 2). Contrary to the morphotype similarity of the newly isolated and the type strain of *F. nolandii*, the sequence similarity points to a closer relationship of our freshwater strain with the marine strain deposited in American Type Culture Collection also as *F. nolandii* (AF293896).

*Legionella* sp. in the cytoplasm of *Filamoeba* sp.

Ultrastructural study of trophozoites and cysts of our *Filamoeba* strain revealed presence of *Legionella*-like bacteria of highly variable shape and length in the cytoplasm and their remnants in phagosomes (Figs 7–10). They were long rod-like bacteria, coccoid forms, and rod-shaped bacteria with large vacuoles or globules (Figs 8–10). Some bacteria released multiple vesicles
DISCUSSION

At a starting point of this study there were parallel isolations of legionellae and an amoeba strain. Standard methods selective for isolating and identifying Legionella spp. from water samples revealed the presence of L. pneumophila, serogroup 2–14 (Hrubá 2009). Since amoebae isolated from the same water source could not be identified with Acanthamoeba or Hartmannella spp. (the most frequently reported hosts of L. pneumophila), the primary task was to determine an amoeba isolate and subsequently to learn more about relationship of this amoeba with legionellae. This approach was justified also by the lack of data about adaptation of L. pneumophila to specific host amoebae and those about multiplication capacity of legionellae in the different amoeba hosts (Albert-Weissenberger et al. 2007).

Although morphological and molecular methods safely assigned the amoeba under study to the genus Filamoeba Page, 1967, species determination resulted premature due to following reasons. Branching pattern of the phylogenetic tree (Fig. 2) gives the impression that newly determined sequence, being a member of the clade with two F. nolandi sequences, might represent this species. However, the sequence AF293896, which is currently the most closely related to the newly determined one, belongs to ATCC 50430 amoeba strain of marine origin. Taking into consideration the difference in salinity between the collecting places of the F. nolandi type strain (freshwater Little Dear Lake, Mi, USA), and ATCC 50430 strain (sediment core 23 nautical miles off coast of Wilmington, NC, USA), the accuracy of identification of ATCC strain is called in question. Unfortunately, the light microscopical and ultrastructural data on ATCC 50430 strain were only briefly summarized in Amaral Zettler et al. (2000). Surprisingly, according to their overview, mitochondria of amoebae of ATCC 50430 strain were only briefly summarized in Amaral Zettler et al. (2000). Surprisingly, according to their overview, mitochondria of amoebae of ATCC 50430 strain possess tubular and branching cristae, which is neither the case of the Filamoeba type strain and F. sinensis we have studied previously (Dyková et al. 2005), nor the case of JIH56 strain. Unlike the previous study that presented phylogenetic position of sequences of F. nolandi and F. sinensis, in the present study, the sequence AF293897 used as an outgroup, is no more denoted as a Filamoeba species. Smirnov et al. (2008) corrected misidentification of ATCC strain 50654 and described this organism as a new species Acramoeba dendroida.

Detection of Legionella sp. in trophozoites of a new Filamoeba strain (JIH56) supplements the list of amoeba hosts of legionellae reported thus far. The temperature tolerance of trophozoites (28–56°C) that surprises in Filamoeba strain was reported also in Hartmannella and Saccamoeba isolates from hot-water systems of hospitals (Rohr et al. 1998). Interestingly, development of a change in temperature tolerance of an amoeba (Acanthamoeba proteus) strain (dependent on endosymbiotic bacteria) was observed in fewer than 200 generations (Jeon and Ahn 1978).
Figs 3–6. Transmission electron micrographs displaying diagnostically important details of the trophozoite ultrastructure of *Filamoeba* strain JIH56. 3 – overview of young trophozoite sectioned at the level of its nucleus. Several mitochondria (arrows), phagosome with amorphous material (P) and one internalized bacterium sectioned longitudinally (arrowhead) can be seen in finely granular cytoplasm; 4 – cell surface of trophozoite. Scale bar: 200 nm; 5 – mitochondrion and its tubular cristae in transverse section. Scale bar: 200 nm; Fig. 6 – mitochondrion in longitudinal section with cristae in parallel arrangement. Scale bar: 500 nm.

Figs 7–10. Transmission electron micrographs of *Filamoeba* trophozoites in early and advanced stage of *Legionella* infection; 7 – cytoplasm of *Filamoeba* trophozoite with rod-like legionellae (arrowheads), transverse sections of legionellae (arrows), two empty phagosomes (P) and part of one replicative phagosome (*); 8 – strongly vacuolized legionellae in phagosome. Scale bar: 1 μm; 9 – two individual phagosomes or two parts of one replicative phagosome containing bacteria and amorphous material. Scale bar: 500 nm; 10 – advanced stage of *Legionella* infection of *Filamoeba* trophozoite. Small membrane vesicles on the surface of bacteria are shed into phagosome. Scale bar: 500 nm.
The intracellular proliferation of Legionella sp. in trophozoites of our Filamoeba strain differed from the ultrastructural pattern documented in axenized amoebae co cultured with L. pneumophila (e.g., Fields et al. 1990). Important details, observed in our material, e.g., formation of RER-free replicative phagosomes agreed with data published by Abu Kwaik (1998b) for L. micdadei infections. The sequence we have determined for Legionella sp. found in Filamoeba strain JIH56 is closely related to L. micdadei, that was described as a third Legionella sp. associated with human pneumonia (Hébert et al. 1980a, b) and is known as the Pittsburgh pneumona agent.

In our Filamoeba strain we expected to determine the same Legionella species that had been diagnosed in water samples (i.e., L. pneumophila). The diagnosis of a different Legionella sp. closely related to L. micdadei was unusual but not unique. Mixed infections of L. micdadei and L. pneumophila that have been reported previously in seven out of 26 patients (Dowling et al. 1983; Muder et al. 1983, 1984) stress the possibility of simultaneous occurrence of two Legionella spp. in the hot water systems or other sources of human legionella infections. The persistence of legionellae in Filamoeba cysts that was proved after their long storage undoubtedly deserves attention.

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