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Interactions of "Limax amoebae" and gram-negative bacteria: Experimental studies and review of current problems

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Free-living amoebae can harbour bacteria inside their cysts giving them a microhabitat and protecting them from disinfectants. The aim of this study was to evaluate the potential importance of "Limax amoebae" as vectors for environmental and nosocomial bacteria in a hospital. It was shown that free-living amoebae are ubiquitous in the investigated hospital, occur syntopically with facultative human pathogens (*Comamonas acidovorans* and *Pseudomonas aeruginosa*) and may serve as hosts not only for these but also for bacteria isolated from clinical specimens (*Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa*); temperature is apparently of crucial importance for the interactions between these microorganisms. Recent studies have shown that "Limax amoebae" apart from acting as protective hosts, may also play a role for the thermotolerance, invasiveness and antibiotic-resistance of bacteria.

Considering also the reduced immune-status of many patients, this "symbiosis" of free-living amoebae and bacteria might still be of underestimated hospital-hygienic importance.

Keywords : Limax amoebae, *Acanthamoeba*, Protozoan vectors, Hospital hygiene

INTRODUCTION

Free-living amoebae have been of medical interest since Culbertson et al. (1958) discovered the ability of one of these Rhizopoda to cause meningoencephalitis in mice and monkeys. Since 1980, when Rowbotham succeeded in isolating pathogenic Legionellae from free-living amoebae, these protozoa have also been considered, in addition to their active pathogenicity, as passive pathogens, harbouring bacteria inside their cysts and protecting them from disinfection. Owing to its extremely resistant cysts the genus *Acanthamoeba* is of particular importance in this respect. Figure 1 gives a review of bacteria, which have been found to survive or even multiply within *Acanthamoeba* spp. However, quite a number of free-living amoebae can act as hosts for bacteria [5].

Our aim was to evaluate the hospital-hygienic relevance of interactions between "Limax amoebae" and potentially pathogenic bacteria. Shower heads in bathrooms of a hospital were investigated on syntopical occurrence of bacteria and free-living amoebae; moreover, bacteria isolated from clinical specimens were tested for their ability to survive within cysts of various amoebae.

MATERIALS AND METHODS

The studies were carried out in the General Hospital of Vienna. Sanitary facilities of this hospital were investigated on the occurrence of free-living amoebae and gram-negative bacteria. The samples were drawn from shower-heads of frequented bathrooms.

Bacteria

The bacterial isolates were obtained by swapping the shower heads with sterile cotton-tipped applicators. The bacteria were grown on various nutrient agars and identi-

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Fig. 1: *Acanthamoeba* spp. as hosts for pathogenic bacteria.

Host	Bacteria	survival/multiplication	Citation site
Acanthamoeba	*Burkholderia pickettii*	survival	MICHEL & HAURÖDER 1997
	Chlamydia pneumoniae	multiplication	ESSIG et al.1997
	Coliforms (incl. *Salmonella typhimurinum*)	survival	KING et al. 1988
	Legionella pneumophila	multiplication, cell-lysis	ROWBOTHAM 1980
	Listeria monocytogenes	multiplication, cell-lysis	LY & MÜLLER 1990
	Mycobacterium leprae	survival	JADIN 1975
	Opportunistical Mycobacteria	survival	KRISHNA-PRASAD & GUPTA 1978
	Pseudomonas aeruginosa	survival	MICHEL et al. 1995
	Vibrio cholerae	multiplication	THOM et al. 1992

fied biochemically by their analytical profile indices.

Amoebae

The amoebae were isolated by the swap-sample-method and cultivated on heat killed *E. coli* seeded Non Nutritive (NN)-agar-plates at 30℃. Thermotolerances (37℃) and growth rates of the isolates were determinated. The amoebae were identified upon morphological characters following PAGE (1991).

Infection of amoebae

In the second part of the study three typical nosocomial agents isolated from clinical specimens (*Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa*) were tested for their ability to survive in the cysts of various amoeba-strains obtained from a previous study in the same hospital. These were a thermophobic *Acanthamoeba lugdunensis*, a thermotolerant *Acanthamoeba rhysodes* and a thermophilic *Hartmannella cantabrigiensis*. The bacterial species were cultured on nutritive agar, transferred into BHI (brain heart infusion), harvested at log-phase and suspended in 0.9% saline. The suspensions were brought to a concentration of 10^8 CFU (colony forming units)/ml by comparison with a McFarland 0.5 $BaSO_4$ standard. 100 μl of these suspensions were spread onto NN-agar-plates.

Axenically grown amoebae were harvested by centrifugation at 1000g/10 min and suspended in amoeba-saline [29]. The amoeba suspensions were calibrated using a Bürker-Türk hemacytometer and brought to a concentration of 10^5 cells/ml. 1 μl of these suspensions was inoculated at the centre of each bacteria seeded NN-agar plate. These cocultures were incubated at 30℃ and 37℃ for 7d. The mature cysts were harvested using sterile cotton-tipped applicators, washed three times in amoeba-saline [29] and transferred into 3% HCl. After having incubated the amoeba-cysts in 3% HCl for 48h in order to kill extracellular bacteria, the intracellular bacteria were reisolated by offering the amoebae heat killed *E. coli* and thus initiating excystment of the amoebae.

RESULTS

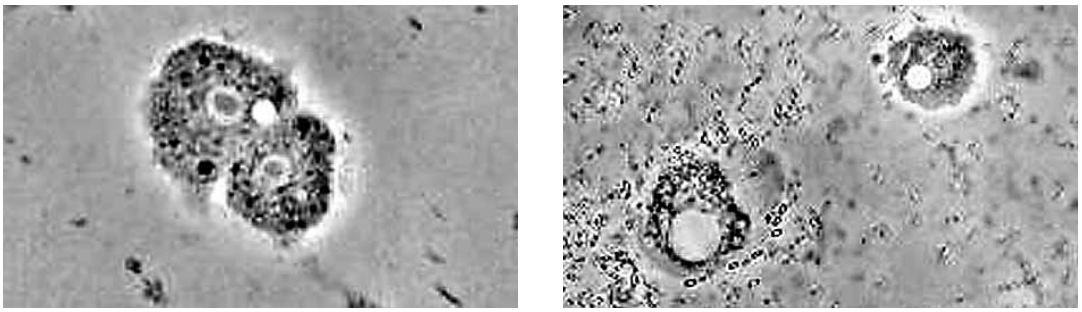
As shown in Figure 2 free-living amoebae were isolated from all investigated habitats. The amoebae were identified as belonging to the genera *Acanthamoeba*, *Echinamoeba*, *Hartmannella* and *Vahlkampfia*. Except one *Acanthamoeba* strain all isolates were able to grow at 37℃.

Two sample sites revealed syntopically occurring bacteria (*Comamonas acidovorans* and *Pseudomonas aeruginosa*). Remarkably the amoebae collected from these sites were heavily infected with the coexisting bacteria (Figure 3b). Up to 30 motile bacteria could be counted per infected cell (trophozoite or cyst). These naturally occurring infections ended lethally for about 50% of the amoebae. The amoebae could not be cultivated in liquid media.

In order to evaluate the ability of different amoebae to act as hosts for nosocomial

agents, three amoeba isolates (*Acanthamoeba lugdunensis*, *A. rhysodes* and *Hartmannella cantabrigiensis*) were infected with different bacteria by the coculture method. Depending on the host amoeba species and on the cocultivating temperature all three bacteria species showed the ability to survive intracellularly. Moreover, there seems to exist an apparent correlation of bacterial survival to the thermotolerance of the host amoeba species (Figure 4).

A. lugdunensis was not an appropriate host, which may be due to its inability to grow at 37℃, as the survival rate of the bacteria was significantly higher at this, for bacterial growth optimal temperature. All three bacteria species could be reisolated from the cysts of *A. rhysodes*, while *H. cantabrigiensis* was an adequate host only for *P. aeruginosa*. Coculture temperature seemed to be of crucial importance for the interactions of these microorganisms.



a **b**

Fig. 3 *Acanthamoeba*: trophozoite in the state of multiplication (a), trophozoites infected with *Comamonas acidovorans* (b)

Fig. 2 Occurrence of free-living amoebae and bacteria on shower heads in bathrooms of the investigated hospital (amoebic growth rates: +-+++)

Sample site	Isolated amoebae			Bacteria
		30℃	37℃	
SS1	*Echinamoeba*	++	++	-
SS2	*Hartmannella*	+++	+	*Pseudomonas aeruginosa*
SS3	*Acanthamoeba* sp. Gr. II	+++	+	*Comamonas acidovorans*
	Vahlkampfia	++	+	
SS4	*E. exundans*	+++	+++	-
SS5	*Acanthamoeba* sp. Gr. II	+++	-	-
	H. vermiformis	+++	+++	

Fig. 4 Survival of *E. coli*, *P. mirabilis* and *P. aeruginosa* in cysts of different amoebae at 30℃ and 37℃

Bacteria	*A. lugdunensis*	*A. rhysodes*		*H. cantabrigiensis*	
	30℃	30℃	37℃	30℃	37℃
E. coli	-	+	+	-	-
P. mirabilis	-	-	+	-	-
P. aeruginosa	-	-	+	-	+

DISCUSSION

All sample sites were positive for amoebae and except one *Acanthamoeba*-strain all isolates exhibited thermotolerance. Similar results were obtained in a previous study in the same hospital, where 16 of 17 sample sites had been amoeba-positive, most of the isolates being thermotolerant or even thermophile (own, unpublished data). Recently Rohr et al. (1998) found thermophilic amoebae in warm-water-systems and on different surfaces of a hospital in Germany. Interestingly that study revealed a comparable species-spectrum to our present study. One may conclude that free-living amoebae do occur ubiquitously in hospitals.

The syntopical occurrence of amoebae and bacteria is of significance from two aspects: On one hand bacteria guarantee a stable amoeba-population, providing the main nutrient source for these protozoa. The sample sites, from which bacteria were isolated, revealed amoebae of the genera *Acanthamoeba*, *Hartmannella* and *Vahlkampfia*. The potential pathogenicity of these isolates was not determined; however, except one *Acanthamoeba* isolate all strains exhibited thermotolerance.

Acanthamoeba is the causative agent of *Acanthamoeba* keratitis and also *Hartmannella* and *Vahlkampfia* were already described as ocular pathogens [2]. In the last years *Acanthamoeba* infections of the ear [15], skin [25] as well as infections of inner organs [18], even one case of amebic osteomyelitis [32] have been described.

On the other hand the cohabitation of free-living amoebae and bacteria contribute to the importance of amoebae as passive pathogens. Nosocomial legionellosis has often been associated with the presence of *Hartmannella vermiformis* [17]. Brieland et al. (1997) could demonstrate that *L. pneumophila* virulence for amoebae is required for maximal intrapulmonary growth of the bacteria and they hypothesize that amoebae may potentiate intrapulmonary growth of *L. pneumophila* by providing a niche for bacterial replication.

The isolated bacteria were identified as *P. aeruginosa* and *C. acidovorans*. Remarkably a majority of the amoebae with coexisting bacteria showed heavy bacterial infections. A study by Michel et al. (1995) revealed syntopically occurring amoebae and *Pseudomonas aeruginosa* in the drinking water system of a hospital and also in this case a majority of the *Acanthamoebae* showed natural occurring *P. aeruginosa* infections. The amoebae enable the intracellularly living bacteria to survive unfavourable conditions (e.g. disinfectants) and can thus act as vectors for the bacteria.

P. aeruginosa and *C. acidovorans*, as cocontaminants of free-living amoebae are of special interest, as both are facultative human pathogens. Recent studies have shown that *Pseudomonas* is one of the predominant organisms associated with corneal ulcers [13, 19]. Also *Comamonas acidovorans* has been described as an ocular pathogen [33]. In the last years the growing importance of *P. aeruginosa* as causing agent for pneumonitis in immunocompromised persons (AIDS) was evaluated [4, 28, 3]. Ali et al. (1995) describe *P. aeruginosa* as causative agent for 6% of respiratory admissions in HIV-positive patients. Moreover, the second part of our study revealed that amoebae obtained from a previous investigation [36] in the same hospital can act as hosts and thus as vehicles for nosocomial bacteria isolated from clinical specimens (*E. coli*, *P. mirabilis* and *P. aeruginosa*) showing an apparent correlation to the physiological abilities of the amoebae, like thermotolerance and growth rate.

Interactions between "Limax amoebae" and bacteria : Current problems

Interactions between "Limax amoebae" and bacteria result in alterations of the amoebae as well as of the bacteria. The exposition of *Acanthamoeba* to virulent *Legionella pneumophila* was shown to induce synthesis of certain amoeba proteins [1]. Moreover the data of Larkin & Easty (1990) indicate that ingestion and metabolism of bacteria enhance virulence and pathogenicity of amoebae. Recently it was suggested that also molecular changes influencing the amoeba virulence are induced by associated bacteria in *Entamoeba histolytica* [9].

On the other hand recent studies have shown that "Limax amoebae" apart from acting as protective hosts, may also play a role for morphological and physiological properties of bacteria. Intra-amoebal grown *Legionella pneumophila* seems to be smaller than in vitro cultured and exhibits enhanced

motility [6]. A study by Thom (1992) revealed that the survival rate of *Vibrio cholerae* is enhanced by intra-amoebal growth.

Susa et al. (1996) report on intra-amoebal growth inducing de novo synthesis of certain *Legionella pneumophila* antigens. *Legionella pneumophila* was also shown to be more invasive for human macrophages after intracellular growth in *Acanthamoeba castellanii* [11]. In a similar study growth of *Mycobacterium avium* in amoebae enhanced entry and intracellular replication in macrophages [12]. It is suggested that survival and intracellular growth of bacteria in protozoa may prime pathogenic bacteria for virulence [5]. There are also studies indicating that bacteria can maintain or even acquire their pathogenicity by these amoeba passages [21].

Intra-amoebal growth apparently also affects the resistance of bacteria to antibiotics. Time survival studies revealed that amoeba-grown bacteria are about 1000 fold more resistant to the activities of rifampicin and ciprofloxacin [8]. Also the sensitivity of *Legionella pneumophila* against different biocides was reduced, when the bacteria had been grown in amoebae [6]. Barker et al. [7] investigated the influence of intra-amoebic growth on surface properties of *Legionella pneumophila* and could demonstrate that intra-amoebic grown *Legionellae*, in contrast to in vitro grown *Legionellae*, contain an outer membrane protein most likely of amoebic origin. They believe that the coating of *Legionellae* with amoebic proteins is involved in the enhanced biocide resistance of intra-amoebic grown bacteria.

Altogether these findings support the assumption that interactions between free-living amoebae and potential human pathogens are rather common and complex and may still be of underestimated hospital-hygienic importance.

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