

Video Article

Discovery of New Intracellular Pathogens by Amoebal Coculture and Amoebal Enrichment Approaches

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URL: <https://www.jove.com/video/51055>

DOI: [doi:10.3791/51055](https://doi.org/10.3791/51055)

Keywords: Immunology, Issue 80, Environmental Microbiology, Soil Microbiology, Water Microbiology, Amoebae, microorganisms, coculture, obligate intracellular bacteria

Date Published: 10/27/2013

Citation: Jacquier, N., Aeby, S., Lienard, J., Greub, G. Discovery of New Intracellular Pathogens by Amoebal Coculture and Amoebal Enrichment Approaches. *J. Vis. Exp.* (80), e51055, doi:10.3791/51055 (2013).

Abstract

Intracellular pathogens such as legionella, mycobacteria and Chlamydia-like organisms are difficult to isolate because they often grow poorly or not at all on selective media that are usually used to cultivate bacteria. For this reason, many of these pathogens were discovered only recently or following important outbreaks. These pathogens are often associated with amoebae, which serve as host-cell and allow the survival and growth of the bacteria. We intend here to provide a demonstration of two techniques that allow isolation and characterization of intracellular pathogens present in clinical or environmental samples: the amoebal coculture and the amoebal enrichment. Amoebal coculture allows recovery of intracellular bacteria by inoculating the investigated sample onto an amoebal lawn that can be infected and lysed by the intracellular bacteria present in the sample. Amoebal enrichment allows recovery of amoebae present in a clinical or environmental sample. This can lead to discovery of new amoebal species but also of new intracellular bacteria growing specifically in these amoebae. Together, these two techniques help to discover new intracellular bacteria able to grow in amoebae. Because of their ability to infect amoebae and resist phagocytosis, these intracellular bacteria might also escape phagocytosis by macrophages and thus, be pathogenic for higher eukaryotes.

Video Link

The video component of this article can be found at <https://www.jove.com/video/51055/>

Introduction

Before the advent of molecular diagnosis, microorganisms present in environmental niches or in clinical samples were often detected by cultivating them on different selective media, mainly on agar in Petri dishes. The phenotype of the bacterial colonies and their metabolic activity then allowed bacterial classification at species level. Broth may also be used to increase the sensitivity of detection. However, both techniques do not allow the recovery of bacteria that grow slowly or not at all on these media. This is the reason why molecular approaches are so widely used nowadays. Nevertheless, detection of DNA provides no clue on the viability of the bacteria. Moreover, contrarily to culture, molecular approaches do not result in a strain that can be further characterized.

Studying pathogens that grow poorly on solid media or that need cells to grow is complicated. Most of these "difficult to grow" bacteria are fastidious intracellular bacteria, often discovered and characterized following large outbreaks as it was the case for *Legionella pneumophila*. This bacterium was characterized following an outbreak that occurred during an American Legion convention. As many as 182 persons were infected and 29 died due to a severe pneumonia^{1,2}. It was later demonstrated that amoebae were the natural hosts of this bacterium and that their presence in the hotel air-conditioning system and water networks was at the origin of the outbreak of the so-called Legionnaire's disease³.

Amoebae are present worldwide and were isolated from soil, air, water and the nasal mucosa of human volunteers (reviewed in ⁴). These "free-living" amoebae are generally dividing autonomously in the environment but may occasionally invade permissive hosts⁵. Amoebae feed on various microorganisms through phagocytosis and subsequent lysosomal digestion by hydrolases⁶. Many facultative or obligate intracellular bacteria are able to resist digestion and thus infect and divide in amoebae as for example *Legionella*, *Chlamydia*-related bacteria or mycobacteria (reviewed in ⁷ and ⁸). Free-living amoebae likely represent an important potential reservoir for intracellular bacteria that have yet not been discovered. This led our group to implement in Lausanne two main techniques, called amoebal coculture and amoebal enrichment, which allowed different groups to isolate several new obligate intracellular microorganisms from various environmental samples⁹⁻¹⁵.

Since amoebae are professional phagocytes grazing on bacteria, a bacterium able to resist phagocytosis and to grow inside these protists might also colonize human phagocytes and be pathogenic towards humans. This was partially demonstrated for some *Chlamydia*-related bacteria, such as *Waddlia chondrophila*. *W. chondrophila* can grow not only in amoebae but also in several cell types such as mammalian epithelial cells, macrophages, and fish cell lines¹⁶⁻¹⁸. The amoebal coculture also appears relevant for detecting intracellular bacteria in clinical samples^{19,20}, including stools which are heavily contaminated with different bacterial species²¹.

Here we describe the major steps of amoebal coculture and amoebal enrichment, including (a) treatment of environmental or clinical samples; (b) the growth of amoebae on axenic media and on a bacterial lawn of *Escherichia coli* and (c) the selection and characterization of intracellular bacteria.

Protocol

1. Amoebal Coculture

1.1 Sample preparation

1. Environmental sample

1. Water samples

Filter the water sample (500 ml to 1 L) through a 0.22 μ m pore size membrane. Then, shake the membrane in Page's amoeba saline medium PAS (120 mg of NaCl, 4 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 142 mg of Na_2HPO_4 , and 136 mg of KH_2PO_4 in 1 L of distilled water).

2. Solid samples

Resuspend solid samples like soil or sand samples and semi-solid samples such as activated sludge in distilled water or PBS and filter them through a 0.22 μ m pore size membrane. Then, shake the membrane in PAS.

3. Samples highly contaminated with endogenous amoebae and protozoa

First sediment the samples by low speed centrifugation (180 x g) for 10 min or filter it through a 5 μ m pore size membrane. Further process the supernatant (respectively Filtrate), as in 1.1.1.

Note: Other decontamination techniques might be used to further decontaminate environmental samples: Heat the sample at 50 °C for 30 min or treat with acidic or basic solutions¹⁴.

2. Clinical sample

Process clinical samples depending on their physico-chemical properties. Filter or centrifuge liquids to remove large impurities. Resuspend solid samples. To use tissues, grind them, for instance by using a dounce homogeniser or glass beads, and lyse the cells to free the intracellular bacteria.

1.2 Amoebae preparation

1. Broth preparation

Prepare the following media: a rich media containing peptone, yeast extract and glucose (PYG; 100 g of proteose peptone, 10 g of yeast extract, 4.9 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g of sodium citrate $\cdot 2\text{H}_2\text{O}$, 0.1 g of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 1.7 g of KH_2PO_4 , 1.97 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 45 g of glucose, and 0.295 g of CaCl_2 in 5 L of distilled water) and a non-nutritive medium such as PAS for *Acanthamoeba* species.

2. Amoebal culture

1. Cultivate amoebae (preferentially *Acanthamoeba castellanii* ATCC 30010 or *A. polyphaga* Linc-Ap1) at 25 °C in cell culture flasks containing 30 ml of PYG medium.
2. Harvest the amoebae by vigorous shaking of the flask and centrifuge the cell suspension for 10 min at 1,500 x g. Wash the pellet twice with PAS medium. Count the cells in a Kova slide and adjust the volume to obtain a suspension of 5×10^5 cells per ml.
3. Transfer the amoebal suspension into microplates. Use 1 ml per well for 12- and 24-well plates, 500 μ l for 48-well plates and 300 μ l for 96-well plates. Incubate the microplate for at least 2 hr at 25 °C. This allows sedimentation and attachment of the amoebae to the bottom of each well.

1.3 Coculture

1. Sample inoculation

1. Inoculate the plate from 2.3.3 with serial dilutions of the sample from 1.1 or 1.2, usually with 10-fold dilution series, starting with 100 μ l of undiluted sample.
2. Centrifuge the microplate at 1,800 x g for 10 min to sediment on the amoebal lawn the microorganisms potentially present in the sample. This increases contact and phagocytosis of microorganisms by amoebae.
3. Incubate the plates for 45 min at 25 °C and wash three times with PAS by replacing the media with fresh PAS. Add 1 ml per well of PAS with or without addition of antibiotics (streptomycin, penicillin, gentamicin and/or vancomycin), depending on the bacterial species that are searched for.
4. Incubate the microplate at 32 °C in a humidified atmosphere to avoid encystment of the amoebae. Observe each well daily with a 20X objective to detect presence of bacteria invading and lysing amoebae.
5. In the case of lysis, perform a subculture on fresh amoebae by inoculating 100 μ l of cocultures to a monolayer of about 10^5 amoebae/ cm^2 . To specifically isolate a given bacterial species, inoculate also specific agar media designed for these bacteria (i.e. BCYE agar for *Legionella* spp.).
6. In the absence of lysis, take 100 μ l of cocultures four to seven days after the first inoculation and inoculate a fresh amoebal culture of 900 μ l in a 24-well plate. If rapid lysis of amoebae is observed without proliferation of bacteria, a virus might be present. In this case, filter the supernatant at 0.22 μ m and use the filtered suspension to infect fresh amoebae.

1.4 Bacterial isolation and characterization

1. Bacterial staining

Perform cocultures directly on glass coverslips in 24-well microplates. Remove the medium and perform staining or immunofluorescence.

1. Modified Romanowsky staining

1. Let the coverslip dry. Immerse the coverslip five times in the fixative solution (2 mg/L Fast Green in methanol).
 2. Immerse the coverslip five times in the staining solution I (1.22 g/L Eosine G in phosphate buffer pH 6.6).
 3. Finally, immerse the coverslip 5 times in the staining solution II (1.1 g/L thiazine in phosphate buffer pH 6.6).
 4. Rinse the sample with distilled water. Let dry and observe by microscopy.
2. Ziehl-Neelsen staining
 1. Let the coverslip dry. Cover the sample with Ziehl fuchsin. Heat the dye with a flame until vapors appear.
 2. Cool at room temperature for at least 5 min and rinse with distilled water. Cover the sample with 3% hydrochloric acid solution in isopropanol for 2 min and rinse with distilled water.
 3. Cover for 30 sec with methylene blue and rinse with distilled water. Let dry and observe by microscopy²².
 3. Gimenez staining
 1. Prepare a basic fuchsin stock solution by mixing 100 ml of 10% basic fuchsin (10 g of basic fuchsin in 100 ml 95% ethanol), 250 ml of 4% aqueous phenol and 650 ml of distilled water. Incubate at 37 °C for 48 hr before use.
 2. Let the coverslip dry and fix by passing it through flame. Cover the sample with freshly filtered basic fuchsin (4 ml of basic fuchsin stock solution in 10 ml of 0.1 M sodium phosphate buffer, pH 7.45) for 2 min.
 3. Rinse the sample with water and incubate in malachite green (0.8% in distilled water) for 10 sec. Rinse again with water and repeat malachite green staining. Rinse again with water. Let the coverslip dry, mount it, and observe by microscopy²³.
 4. Immunofluorescence
 1. Fix the coverslip by incubating in methanol for 5 min or with paraformaldehyde 4% for 10 min.
 2. Wash three times with PBS and incubate for 2 hr in blocking solution (5% BSA, 0.1% Saponin in PBS) at room temperature.
 3. Incubate the coverslip for 1 hr in blocking solution containing antibodies raised against the microorganism of interest.
 4. Wash again three times in PBS and incubate for 1 hr with a secondary antibody directed against the primary antibody and linked to a fluorophore. Wash three times with PBS, mount the coverslip and observe by fluorescence microscopy.

2. DNA detection by PCR

Extract DNA from 100 to 200 µl of amoebal coculture. Detect microorganisms with universal primers targeting the 16S rRNA gene or specific primers for species of interest such as mycobacteria²⁴, legionella²⁵ or members of the *Chlamydiales*²⁶.

2. Amoebal Enrichment

2.1. Sample preparation

Resuspend solid and semi-solid samples in PAS by vortexing. Centrifuge the suspension at low speed (180 x g) for 10 min. This allows enrichment of free-living amoebae in the pellet. The supernatant can be used for amoebal coculture and the pellet for amoebal enrichment²¹.

2.2. Medium preparation

1. Add 1.5 g of agar to 100 ml of PAS and autoclave the medium 15 min at 121 °C. Pour the warm medium in Petri dishes and let solidify at room temperature.
2. Grow *Escherichia coli* (ATCC 25922) in LB or thioglycolate broth overnight at 37 °C. Wash the bacteria twice with PBS and resuspend them in PAS medium. Dilute 10x in PAS and spread 2-3 ml of this dilution on a PAS agar plate and let dry.

2.3. Sample inoculation

Add a drop of sample (or a piece of filter) on one side of the plate and let it flow over the Petri dish to form a line in the center of the dish.

2.4. Amoebal growth and amoebal subculture

1. Observe the Petri dish daily. If an amoebal migration front is detected, cut out a small piece of agar at the migration front and inoculate a fresh NNA Petri dish covered with a lawn of *E. coli*.
2. Repeat the reinoculation several times depending on the purity of the sample, in order to have a pure culture of a given amoebal strain.

2.5. Amoebae and bacteria characterization

1. Scrape the cells and resuspend them in PAS.
2. Extract DNA and determine the identity of amoebae and/or bacterial endosymbionts by PCR and sequencing (16S rRNA amplification for bacteria, resp. 18S rRNA for the amoebae and sequencing, for example).
3. Use these scraped cells in PAS to inoculate fresh amoebae and perform an amoebal coculture to detect bacteria possibly present in the sample.

Representative Results

Using amoebal coculture and amoebal enrichment, a whole range of environmental and/or pathogenic bacteria were discovered (**Table 1**).

Amoebal coculture was used by our group and others to analyze environmental samples, water treatment plants and water distribution systems. A broad range of microorganisms could be isolated with this technique. The most common bacteria isolated by amoebal coculture are members of the *Mycobacterium* genus that could be recovered from water treatment plants and from water networks^{13,14,24,27,28}. *Legionella* and α-

proteobacteria species could also be isolated from water treatment plants and from hospital water networks^{14,24,28-31}. Several *Chlamydia*-related species were also isolated from river water and water treatment facilities, as for example *Estrella lausannensis* (Figures 2B-C)^{12,15,32,33}.

Using amoebal coculture, different giant viruses were discovered, such as Mimivirus, Marseillevirus and Lausannevirus³⁴⁻³⁶. These viruses are all able to infect and multiply within amoebae and present an eclipse phase typical of their viral lifestyle. The mimivirus is considered as a mild human lung pathogen since it was implicated in an accidental infection of a laboratory technician who suffered from pneumonia³⁷. Potential pathogenicity of other giant viruses still needs to be investigated.

Amoebal enrichment was often used in parallel to amoebal coculture. Thus, when investigating a water treatment plant system and the downstream water network, 25 different amoebal strains have been detected, of which 12 corresponded to new species¹⁴. Amoebae were present at every step of water purification and distribution, indicating a resistance of these protists to ozonation and chlorination. Intracellular bacteria could also be detected in these amoebae, showing the importance of indigenous amoebae in the transmission of intracellular bacteria¹⁴. In another study, amoebae could be isolated from the water distribution system of a hospital²⁴. A large majority of the strains isolated in this study corresponded to *Hartmannella vermiformis*, which is naturally able to survive at relatively high temperatures. Several bacteria, such as *Legionella pneumophila* could be detected in the indigenous amoebae²⁴. Another example of bacteria found in a specific amoeba is *Parachlamydia acantamoebae*. This *Chlamydia*-related bacterium was isolated from nasal mucosa of female volunteers by amoebal enrichment (Figure 2A)⁹ and is a potential agent of pneumonia³⁸. This again shows the importance of amoebae in the maintenance and dispersion of bacterial pathogens that might be especially pathogenic for immunocompromised patients³⁹.

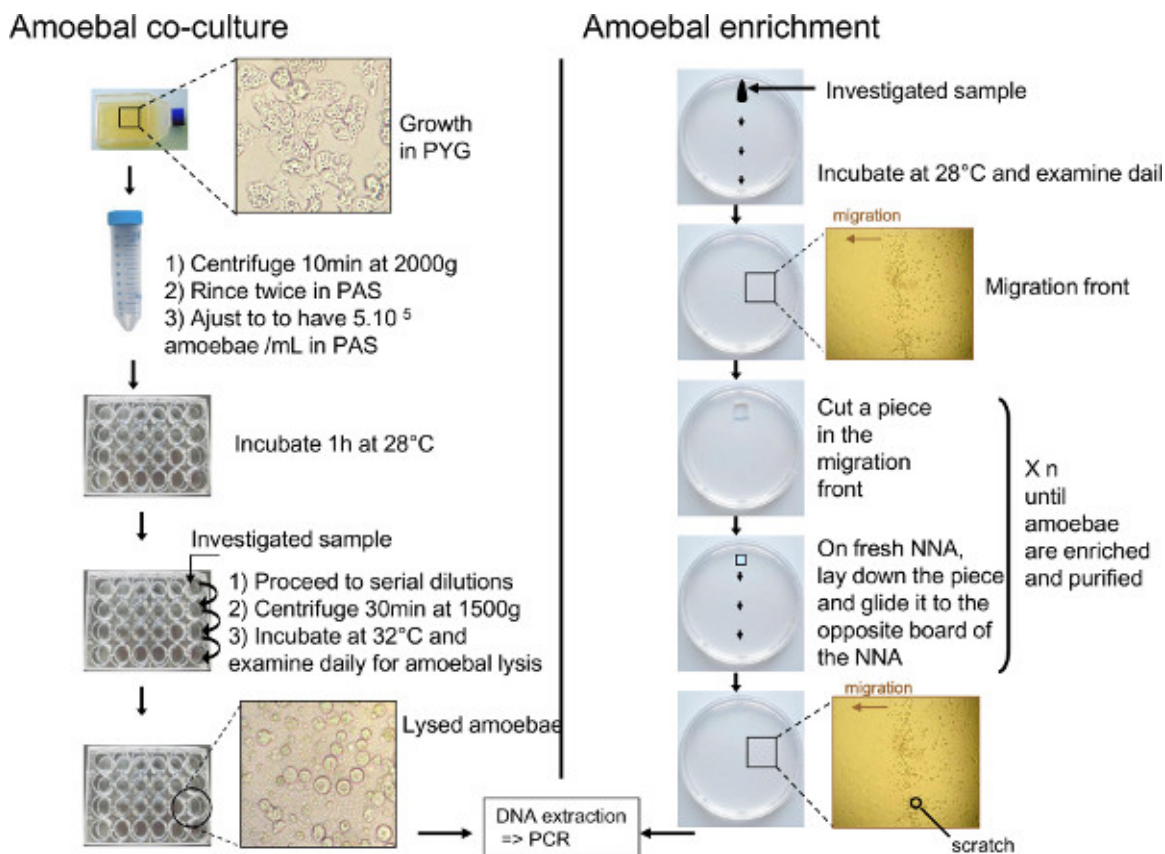


Figure 1. Outline of amoebal coculture and amoebal enrichment describing the important steps of these two techniques. (Adapted from ⁴⁰). [Click here to view larger figure.](#)

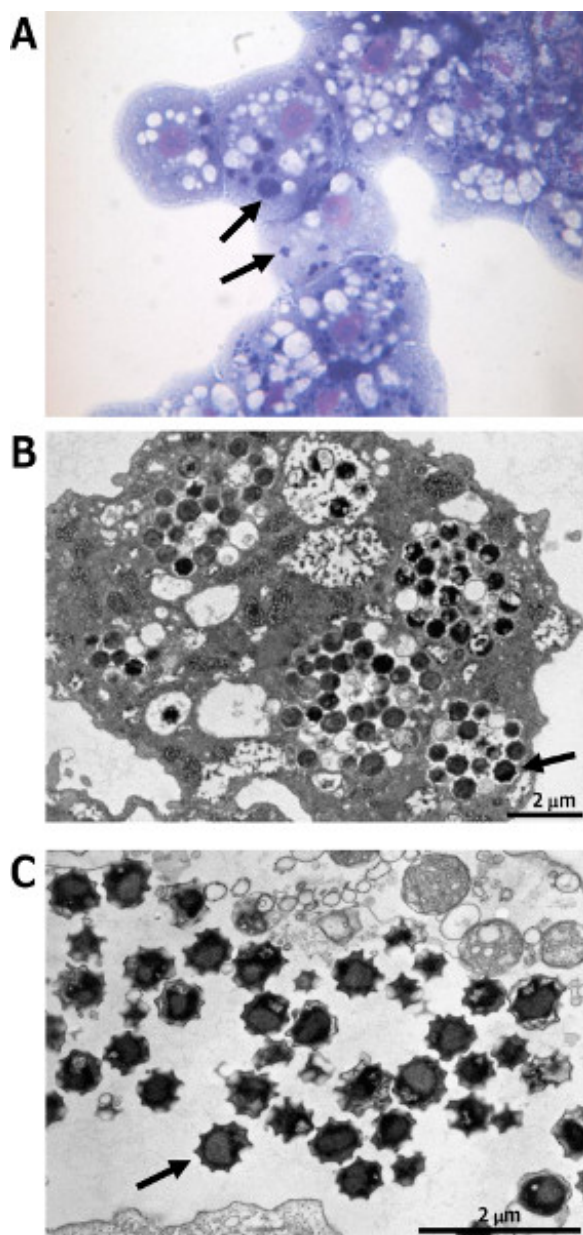


Figure 2. Examples of bacteria discovered by amoebal coculture and their observation with different staining methods. A) Staining of *Parachlamydia acanthamoebae* Hall's coccus infecting amoebae with modified Romanowsky method 24 hr after infection, with a magnification of 1,000X. Bacteria are stained in blue (arrows). B-C) Electron microscopy of *Estrella lausannensis* showing the typical star morphology of elementary bodies (arrows).

Class	Species examples	Reference
<i>α</i> -proteobacteria	<i>Odyssella thelassonicensis</i>	10
<i>b</i> -proteobacteria	<i>Burkholderia cepacia</i>	36
<i>g</i> -proteobacteria	<i>Legionella drancourtii</i>	26
Chlamydiae	<i>Estrella lausannensis</i>	30
Flavobacteriae	<i>Amoebophilus asiaticus</i>	37
Actinobacteria	<i>Mycobacteria spp.</i>	38

Table 1. Examples of bacteria from different classes discovered by amoebal coculture.

Discussion

Amoebal coculture and amoebal enrichment are efficient methods that allowed the isolation of many new bacterial and amoebal species. Results obtained with these methods confirm the ubiquitous presence of both amoebae and amoeba-resisting bacteria in the environment, and most interestingly in manmade water networks that are considered to be controlled by chemical treatments such as chlorination and ozonation. Amoebal coculture and amoebal enrichment are essential tools to isolate and cultivate these potentially pathogenic microorganisms and to obtain strains in pure culture in order then to further study their biology and pathogenicity. Recently, this method was adapted for high-throughput isolation of giant viruses⁴¹. Similarly, amoebal coculture might be automated and used as a routine technique to test microbiological quality of environmental samples and manmade water systems, such as drinking water.

Amoebal coculture can be performed with different species of amoebae. We usually prefer to use *Acanthamoeba castellanii* or *A. polyphaga* since they are less prone to encystment than *Hartmannella vermiformis* and since they exhibit a relatively large host spectrum. Other species might be used but the protocol and broth should be adapted. It has been recently demonstrated that *A. lenticulana* (ATCC 30841) can be used in the same conditions as *A. castellanii* or *A. polyphaga* but has different susceptibility to infection⁴². To prevent encystment, it is important to use a relatively low incubation temperature (below 30 °C) and to maintain a humidified atmosphere.

Noteworthy, the sustained replication of amoebal symbionts will not necessarily lead to amoebal lysis and when specifically looking for symbionts, screening by microscopy and/or PCR should be systematically performed. To avoid lysis or detachment of amoebal cells due to bacterial overgrowth, it is useful to perform inoculation of heavily contaminated samples using 10-fold serial dilutions.

Nevertheless, these techniques have limitations. Due to the use of a single amoebal species, the amoebal coculture might not allow the isolation of bacteria that use as reservoir another amoebal species. Moreover, such specific amoebal species might be unable to proliferate on *E. coli* lawns and may be missed by amoebal enrichment. Depending on the properties of the bacteria or amoebae investigated, it might be necessary to test different media, different amoebal species and different bacterial species to feed amoebae (*Enterobacter doacae* and some *Pseudomonas* strains are good alternatives). Bacterial contamination of amoebae or media used for amoebal coculture may occur and may lead to false positive results. A negative control is thus mandatory to avoid such false positive results.

In conclusion, amoebal coculture and amoebal enrichment are two complementary approaches that represent interesting tools to specify the ecology and biodiversity of free-living amoebae and of amoebae-resisting bacteria. These techniques allow the discovery of many new species, including amoebae, bacteria and giant viruses, forming the basis for future studies to investigate the pathogenicity of these novel microorganisms.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

We thank Pr. Bernard La Scola for helpful technical advices and interesting discussion on amoebal coculture and amoebal enrichment. We also thank Dr Vincent Thomas for his help in implementing the technique in our laboratory.

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