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Importance of amoebae as a tool to isolate amoeba-resisting microorganisms and for their ecology and evolution: the Chlamydia paradigm.

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Running title :Discovering new pathogens

Summary

Free-living amoebae are worldwide distributed and frequently in contact with humans and animals. As cysts, they can survive in very harsh conditions and resist biocides and most disinfection procedures. Several microorganisms, called amoeba-resisting microorganisms (ARMs), have evolved to survive and multiply within these protozoa. Among them are many important pathogens, such as legionella and mycobacteria but also several newly discovered *Chlamydia*-related bacteria, such as *Parachlamydia acanthamoebae, Estrella lausannensis, Simkania negevensis* or *Waddlia chondrophila* whose pathogenic role towards human or animal are strongly suspected.

Amoebae represent an evolutionary crib for their resistant microorganisms since they can exchange genetic material with other ARMs and develop virulence traits that will be further used to infect other professional phagocytes. Moreover, amoebae constitute an ideal tool to isolate strict intracellular microorganisms from complex microbiota, since they will feed on other fast growing bacteria such as coliforms potentially present in the investigated samples. The paradigm that ARMs are likely resistant to macrophages, another phagocytic cell and that they are likely virulent towards humans and animals is only partially true. Indeed, we provide examples of the *Chlamydiales* order that challenge this assumption and suggest that the ability to multiply in protozoa does not strictly correlate with pathogenicity and that we should rather use the ability to replicate in multiple and diverse eukaryotic cells as an indirect marker of virulence towards mammals. Thus, cell-culture based microbial culturomics should be used in the future to try to discover new pathogenic bacterial species.

1. Introduction

During the last decade, growing interest for amoebae and amoeba-resisting microorganisms (ARMs) led to a bulk of research (reviewed in (Greub and Raoult, 2004; Lamoth and Greub, 2010)). Amoebae have been successfully used to discover several new giant viruses (Boyer et al., 2009; Thomas et al., 2011) and new fastidious bacterial species (Thomas et al., 2006a; Lienard et al., 2011a). Here we will review the most recent knowledge gathered on (i)

free-living amoebae (FLA), (ii) amoeba-resisting microorganisms, (iii) the role of amoebae in the ecology and evolution of amoeba-resisting bacteria and (iv) the usefulness of amoebae as a tool to discover new pathogens.

Finally, we will challenge the paradigm that amoebae are preferentially isolating pathogens (Greub and Raoult, 2004), since it appears that (i) resistance to amoebae is not a prerequisite for pathogenicity and (ii) many ARMs are harmless environmental species.

2. Free-living amoebae

Free-living amoebae are ubiquitous protists that are distributed worldwide and have been isolated from soil, water and air (Rodriguez-Zaragoza, 1994; Schuster and Visvesvara, 2004; Trabelsi et al., 2012). They are usually found in biofilms and at the interfaces between water and soil, water and air as well as water and plants where nutrients are abundant and where conditions of light, temperature, oxygenation and pH are compatible with their growth. Various free-living amoebae such as *Acanthamoeba, Hartmanella* or *Naegleria* have also been recovered from cooling towers, hydrotherapy baths, swimming pools and hospital or domestic water networks (Corsaro and Greub, 2006; Thomas et al., 2006b; Thomas et al., 2008; Thomas and Ashbolt, 2011). Thus, humans and animals are frequently in contact with free-living amoebae that can be pathogenic by themselves or that may harbour pathogenic microorganisms.

Most free-living amoebae exhibit two developmental stages: the trophozoite, a feeding form that multiply by binary fission and the cyst, a dormant form observed under adverse conditions of temperature, pH, moisture or in the absence of nutrients (Rodriguez-Zaragoza, 1994). Some amoebal species, such as *Naegleria* spp. also exhibit a third flagellate form (Marciano-Cabral, 1988). Cysts are able to survive in very harsh conditions and thus can resist biocides and most disinfection processes (Greub and Raoult, 2003a; Thomas et al., 2008). They can revert to their active form when better growth conditions become available.

Trophozoites feed on various microorganisms that are engulfed by phagocytosis, before entering the endocytic pathway and being digested in phagolysosomes. Some

microorganisms however have developed strategies to resist digestion and have acquired the ability to survive or even to multiply within free-living amoebae (Greub and Raoult, 2004). They are called amoeba-resisting microorganisms (ARMs) and include bacteria, fungi and viruses.

3. Amoeba-resisting microorganims

3.1 Bacteria

Extensive reviews listing amoeba-resisting bacteria (ARB) that have been recovered by amoebal co-culture from environmental samples or in water systems, have been published elsewhere (Greub and Raoult, 2004; Lienard and Greub, 2011). Since then, the list has grown even longer with the discovery of *Estrella lausannensis*, *Reyranella massiliensis* (Lienard et al., 2011a; Pagnier et al., 2011) and several other amoebae-resisting bacteria, all isolated from environmental water samples (Corsaro et al., 2010b; Corsaro et al., 2013b). The diversity of these ARB is tremendous particularly with regard to their lifestyle and to their pathogenicity. Indeed, they can be either facultative or obligate intracellular organisms like *Legionella* spp. or *Chlamydia*-related bacteria (Greub and Raoult, 2002b), respectively. Moreover, free living organisms such as *Acinetobacter* spp. or *Pseudomonas* spp. have also been recovered by amoebal co-culture (Thomas et al., 2006b; Thomas et al., 2008). Their way of interacting with their host may be highly variable and some, such as *Candidatus* Amoebophilus asiaticus represent true endosymbionts maintaining a long-term stable relationship with their host (Horn et al., 2001), whereas others, such as *Legionella drancourtii,* are clearly cytopathic and may rapidly lyse their amoebal host (La Scola et al., 2004). Finally, some ARB may be lytic or endosymbiotic according to specific environmental conditions such as temperature (Birtles et al., 2000; Greub et al., 2003b).

The pathogenicity of ARB is also variable, ranging from recognized human pathogens (*Mycobacterium avium, Vibrio cholerae),* to completely harmless species (*Azorhizobium* spp.*, Muricoccus roseus).* Recent publications also demonstrate that several newly discovered ARB such as *Waddlia chondrophila* or *Parachlamydia acanthamoebae* are emergent human

or animal pathogens (Greub, 2009; Lamoth and Greub, 2010; Baud and Greub, 2011; Baud et al., 2013)

In addition to those directly recovered from amoebae by amoebal co-culture or amoebal enrichment (Lienard and Greub, 2011), a number of bacteria have demonstrated *a posteriori* their ability to survive or even to replicate in amoebae. It is, for example, the case of *Legionella pneumophila* that was first isolated from lungs of infected patients by inoculation to guinea pigs (McDade et al., 1977) and was only later shown to be able to grow in *Acanthamoeba* and *Naegleria* (Rowbotham, 1980), thus enlightening the role of this protist in *Legionella* dissemination (Rowbotham, 1986). Similarly, *Waddlia chondrophila* was first isolated from an aborted bovine fetus (Henning et al., 2002) and later shown to grow in *Acanthamoeba castellanii* (Michel et al., 2004; Goy and Greub, 2009), which suggested that amoebae from water sources could be a reservoir for the bacteria (Codony et al., 2012).

3.2 Viruses and fungi

A giant virus, hosted in free-living amoebae, has first been observed in 1992 during an outbreak of pneumonia (La Scola et al., 2003). Mimivirus (microbe mimicking virus) consists of a 400 nm particle surrounded by an icosahedral capsid and, because of its size, was originally mistaken for an intracellular bacteria and initially named “Bradford coccus”. It is a member of the NucleoCytoplasmic Large DNA Virus group (NCLDV) and its genome of 1.2 Mb encodes genes implicated in metabolic processes and in protein synthesis thus endowing the virus with a putative autonomy (Claverie et al., 2009; Yamada, 2011). Positive serology, viral DNA and very recently the viral particle itself were documented in patients with pneumonia, suggesting the potential role of this virus as a respiratory pathogen (Vincent et al., 2010; Vanspauwen et al., 2012; Mueller et al., 2013; Saadi et al., 2013)

Several other giant viruses have been isolated from environmental samples, including Marseillevirus (Boyer et al., 2009), Lausannevirus (Thomas et al., 2011) and the recently described Pandoravirus, with a size of about one micrometer, that can be observed with an optical microscope (Philippe et al., 2013). The prevalence of these viruses in the

environment as well as their potential pathogenicity towards humans or animals remain to be determined (Popgeorgiev et al., 2013).

Other viruses such as enteroviruses or adenoviruses are able to infect free-living amoebae suggesting that the protists may act as vehicles and reservoirs for these microorganisms, playing a role in their dispersal (Danes and Cerva, 1981; Scheid and Schwarzenberger, 2012).

Finally, protozoan parasites such as *Cryptosporidium parvum,* responsible of intestinal diseases or the soil fungus *Cryptoccoccus neoformans,* causing severe meningitis in immunocompromised patients, have also been shown to replicate in *Acanthamoeba* sp (Steenbergen et al., 2001; Scheid and Schwarzenberger, 2011), illustrating the wide biodiversity of microorganisms able to resist amoebal destruction.

4. Roles of amoebae in the ecology and evolution of ARMs

Amoebae are considered to play multiple important roles in the ecology and evolution of their resistant microorganisms (Greub and Raoult, 2004; Greub, 2009). These roles are summarized in Table 1.

1) Replicative niche

Amoebae can provide a replicative niche furnishing optimal conditions for bacterial multiplication. Several ARMs are restricted to one specific amoebal species and are unable to infect other protists (Birtles et al., 2000; Horn et al., 2000). Interestingly, several microorganisms able to grow in amoebae are also able to survive in macrophages. Moreover, *Legionella* mutants defective for growth in amoebae were also defective for growth in macrophages, supporting the paradigm that amoebae could represent an evolutionary crib for these microorganisms (Gao et al., 1997) (see below)

2) Widespread reservoir

Amoebae infected with highly adapted bacteria such as *Legionella pneumophila* or *Parachlamydia acanthamoebae* can be filled with hundreds or thousands of bacteria (Rowbotham, 1986; Greub et al., 2004a). *Mycobacterium* spp. or *Listeria monocytogenes* are

also able to multiply within amoebae, though to a lesser extent (Adekambi et al., 2004; Akya et al., 2009). Moreover, during an investigation of water samples from a hospital water network, a strong association was demonstrated between the presence of amoebae and the presence of *Legionella* ($p<0.001$) or *Mycobacteria* ($p=0.009$), suggesting that protists indeed play an important role of reservoir for these bacteria (Thomas et al., 2006b). A more recent study by Garcia et al. also demonstrate the presence of pathogenic ARMs such as *Legionella, Mycobacteria* or *Pseudomonas* in free-living amoebae isolated from reservoirs and water treatment plants. Amoebae can also be reservoir for viruses. Indeed, several giant viruses have recently been isolated from various water samples in various locations (Yoosuf et al., 2012; Aherfi et al., 2013; Boughalmi et al., 2013; Philippe et al., 2013) and new viral strains and species have been isolated by amoebal co-culture (Boyer et al., 2009; Thomas et al., 2011). The implication of such a widespread reservoir for ecology and public health has still to be defined.

3) Protective armour

ARMs are potentially able to survive during amoebal encystment and thus could use protists as protective armour to avoid destruction by chlorine or other biocides (Steinert et al., 1998; Kahane et al., 2001; Coulon et al., 2010). Consequently, amoebal cysts are playing an important role in the persistence of microorganisms in the environment explaining the low efficiency of biocides and sterilization procedures and the occurrence of pseudo-outbreaks in the presence of amoebal cysts (Greub and Raoult, 2003a; Storey et al., 2004; Dupuy et al., 2014).

4) Dissemination mode

Aerosols are probably the predominant way of transmission of ARMs to humans as clearly demonstrated for *Legionella pneumophila* (Hart and Makin, 1991). Bacteria may be transmitted to their final host either free, trapped in their Trojan amoebal host (see below), or tightly packed in vesicles containing thousands of bacteria (Rowbotham, 1986; Greub and Raoult, 2004). Such expelled vesicles, that represent a way used by amoebae to postpone

their own lysis, has been reported for *Legionella* (Berk et al., 1998), *Parachlamydia* (Greub and Raoult, 2002b) and *Burkholderia* (Inglis et al., 2000).

5) Trojan horse

Bacteria can use amoebae as Trojan horses to invade human or animal organisms. One of the best examples of enhanced entry of an ARB thank to the presence of amoebae was provided by Cirillo *et al*. using a mouse model of *Mycobacterium avium* infection. The number of CFU/ml present in the mice gut was increased in presence of increasing numbers of amoebae (Cirillo et al., 1997). In addition, it is noteworthy that *Parachlamydia acanthamoebae* is endosymbiotic for *Acanthamoeba polyphaga* at 25-30°C while it is lytic at 32-37°C (Greub et al., 2003b), a characteristic that could allow the bacteria to safely enter a human or animal organism when amoebae colonize the nasal mucosa and to lyse their « Trojan horse » when they reach the lower respiratory tract where the temperature is higher and where they will cause infection. Thus, their Trojan horses are protecting the internalized bacteria from the first line of defenses of the final human or animal host.

6) Gene exchange

Being the ecological niche of a large variety of microorganisms, amoebae represent an agora, where horizontal gene transfer events occur between different ARMs and between them and their amoebal host (Ogata et al., 2006; Saisongkorh et al., 2010; Thomas and Greub, 2010; Gimenez et al., 2011; Bertelli and Greub, 2012; Lamrabet et al., 2012; Gomez-Valero and Buchrieser, 2013). For example, *Chlamydiales* and *Rickettsiales* both possess a rare transport protein allowing energy parasitism by enabling the import of host cell ATP in exchange for ADP. Phylogenetic analyses suggest that this ATP/ADP translocase has evolved from a *Chlamydiae* ancestor and has been acquired by *Rickettsiae* via lateral gene transfer (Greub and Raoult, 2003b; Schmitz-Esser et al., 2004). In addition a similar Type IV secretion system (T4SS) encoding all genes necessary for gene conjugative transfer is present in the genome of these two ARB (Greub et al., 2004b; Ogata et al., 2006). Finally, it is probable that genetic exchanges have shaped the ARMs to confer them selective advantages for intracellular life in various hosts, which also explains why sizes of ARB

genomes are generally larger than the genome size of a bacteria strictly adapted to humans (Moliner et al., 2010).

7) Selection of virulence traits

Amoebae could also represent a training ground allowing selection of virulence traits (Cirillo et al., 1994; Cirillo et al., 1997; Greub and Raoult, 2004). Thus, as compared to *in vitro*-grown *Legionella*, bacteria grown in amoebae are far more resistant to harsh conditions such as high temperature, high osmolarity or acidity (Barker and Brown, 1994). In addition, *Legionella* grown within a protozoan host display an increased infectivity for mammalian host cells *in vitro* (Cirillo et al., 1999) and are also more infectious for mice *in vivo* (Cirillo et al., 1994; Brieland et al., 1997). Interestingly, this increased infectivity has been related to a specific phenotype called “MIF” (mature intracellular form) (Garduno et al., 2002; Greub et al., 2004a). Similarly, entry and intracellular replication are enhanced when *Mycobacterium avium* are grown in amoebae as compared to axenically grown bacteria, with increased virulence observed in a mouse model of infection (Cirillo et al., 1997).

8) Adaptation to macrophages

For ARMs, amoebae represent an evolutionary crib in which they can develop strategies helping them to survive the microbicidal effectors of other professional phagocytic cells such as macrophages. This adaptation to macrophages has been particularly well studied for *Legionella,* both at the cellular and molecular levels. At the cellular level, it was demonstrated that the *Legionella*-containing phagosome is associated with the rough endoplasmic reticulum both in amoebae and in macrophages and that this association sustains bacterial multiplication (Swanson and Isberg, 1995; Abu Kwaik, 1996). At the molecular level, the same genes are used for multiplication in amoebae and in macrophages (Segal and Shuman, 1999; Stone et al., 1999) and mutants defective for intracellular growth in amoebae are also unable to replicate in macrophages (Gao et al., 1997). The similar strategies used to avoid destruction by professional phagocytic cells are also observed for other microorganisms such as *Cryptoccoccus neoformans* or *Mycobacterium avium* (Cirillo et al., 1997; Steenbergen et al., 2001).

5. Amoebae as a tool to discover new ARMs

Obligate intracellular microorganisms cannot grow on axenic media and thus certainly remain largely undetected by conventional diagnostic procedures even though some of them are significant human pathogens. Amoebae represent an interesting tool for isolating such intracellular microorganisms by amoebal co-culture and amoebal enrichment (Figure 1), two techniques that are described in detail in a recent book chapter by Lienard and Greub (2011) and whose advantages and limitations are summarized in Table 2.

In amoebal co-culture, the protist is used as a eukaryotic host able to sustain multiplication of ARMs. The technique can be applied to environmental samples in order to investigate the source of an outbreak. It could also be applied to clinical samples as a diagnostic tool for patients with infections of unknown etiology, even in samples physiologically exhibiting a huge microbial diversity such as nasal secretions, feces and sputa.

Amoebal enrichment allows isolation of the protist (potentially containing an endosymbiont), by multiple passages on non-nutritive agar (NNA) covered with *E. coli*. Practically, amoebae will feed on bacteria and will constitute a migration front of trophozoites that can be enriched by subsequent passages on new NNA.

With both techniques, DNA is finally extracted and the ARM can be identified using either specific primers targeting various ARMs or universal primers amplifying the 16S rRNA gene that can be further sequenced (Lienard and Greub, 2011). Such molecular identification may be completed by FISH, electron microscopy, immunofluorescence with specific antibodies, or even by full genome sequencing.

6. Challenging the concept of ARM as pathogens

6.1 Generalities

Given the role of amoebae as an evolutionary crib for the selection of virulence traits and as a training ground for resistance to macrophages (see above), these protists are generally considered as an ideal tool to isolate yet undiscovered microorganisms and more precisely

new important human or animal pathogens. However, we challenge here the idea that amoebae may be used to preferentially isolate pathogenic bacteria based on two facts:

- First, most ARMs discovered to date are either non pathogenic or have limited impact on human/animal health. Thus, among hundreds of amoeba-resisting microorganisms (Lienard and Greub, 2011), only few (such as *Legionella* or *Mycobacteria* among others) are established pathogens. Of course, since most ARMs are fastidious intracellular bacteria, (or viruses), lack of known pathogenicity does not necessarily mean lack of pathogenicity.
- Secondly, several intracellular bacteria, such as *Bartonella*, *Rickettsia* or *Chlamydia* spp, have only limited capacities to grow within amoebae despite being effective pathogens and able to easily multiply in epithelial or endothelial cells. Furthermore, it has been recently demonstrated that, in the case of *Mycobacterium bovis*, amoebae do not support the survival of the virulent bacteria and even contribute to its inactivation and thus cannot represent an environmental reservoir for this microorganism (Mardare et al., 2013).

Moreover, researchers paid more attention to pathogenic ARMs, using specific detection tools, due to the health risk linked to their presence in amoebae. In addition, detection of non-pathogenic ARMs is under-reported. Please note that most ARMs are pathogenic towards amoebae inducing their lysis: here the word “pathogen” refers to pathogenicity demonstrated (or highly suspected) against vertebrates. In this perspective, it is important to stress that many environmental ARMs do not grow or only poorly grow at temperatures of warm blooded vertebrates, thus limiting their pathogenicity.

6.2 *Chlamydiales* as examples

The *Chlamydiales* order is constituted of obligate intracellular bacteria comprising harmless symbionts of protozoa as well as important human and animal pathogens. This clade is currently divided in 9 family-level lineages (*Parilichlamydiaceae, Piscichlamydiaceae,*

Clavichlamydiaceae, Chlamydiaceae, Simkaniaceae, Rhabdochlamydiaceae, Waddliaceae, Criblamydiaceae and *Parachlamydiaceae*) (Stride et al., 2013a), but a very recent publication by Lagkouvardos et al. (2014) suggests a tremendous ecological diversity at the species, genus and family levels (181 putative families), with the marine environment being the source of the majority of the newly identified *Chlamydiales* lineages .

Several members of the *Chlamydiales* order that were isolated from amoebae or by amoebal co-culture, such as *Parachlamydia amoebophila, Neochlamydia hartmanellae* or *Criblamydia sequanensis* are probably environmental harmless species since none of the studies performed so far could identify these organisms in human infections. However, several serological and molecular hints suggest a potential role of *P. acanthamoebae* in pneumonia (Ossewaarde and Meijer, 1999; Marrie et al., 2001; Greub et al., 2003c; Casson et al., 2008) despite the limited replication of this bacteria in pneumocytes and lung fibroblasts (Casson et al., 2006). A limited replication was also observed in macrophages, likely due to the induced apoptosis of these major innate immune cells (Greub et al., 2003a; Greub et al., 2005; Roger et al., 2010). The restricted growth of *P. acanthamoebae* and *P. amoebophila* in insect cells is also related to their inability to inhibit apoptosis (Sixt et al., 2012), which appeared to be an essential step for successful development of most obligate intracellular bacteria.

A recent work by Rusconi *and Greub(2013)*, demonstrated that, contrarily to *Chlamydiaceae*, several *Chlamydia*-related bacteria possess a functional catalase, an enzyme likely important for survival in phagocytic cells such as macrophages. The total absence of this enzyme in *Chlamydiaceae* might be one reason why the macrophage and amoebal environments are hostile to these bacteria (Wirz et al., 2008; Beagley et al., 2009). Indeed, *Chlamydiaceae* recognized as human or animal pathogens (*C. pneumoniae, C. abortus* and *C. trachomatis)* are not able to grow in amoebae or only to a very limited extent (Essig et al., 1997; Wirz et al., 2008; Coulon et al., 2012) while they readily multiply in epithelial or endothelial cells where they are able to inhibit apoptosis (Ying et al., 2007; Sharma and Rudel, 2009). In monocytes, macrophages and their derived cell lines, *Chlamydia* replication is very limited and the cells are resistant to apoptosis. Even though bacteria survive in these cells and are

even metabolically active, they differentiate in an enlarged, dormant form called aberrant bodies and no infectious Elementary Bodies (EBs) are produced (Beagley et al., 2009). Indeed, these dormant forms of the bacteria, viable but non cultivable, are associated with persistence and may lead to chronic inflammatory diseases (Beatty et al., 1994; Hogan et al., 2004). The same observation has been made in insect cells where *C. trachomatis* EBs are able to enter, differentiate into Reticulate Bodies (RBs) and replicate but where bacteria do not lyse their host cells and where no infectious progeny is produced (Elwell and Engel, 2005).

From the examples described above we can conclude that, although amoebae and macrophages have similar microbicidal machineries, the ability to multiply in amoebae does not necessarily implicate an ability to grow in other phagocytic cells such as macrophages. Furthermore, the pathogenicity towards mammals is not directly correlated to the ability of a given species to grow in amoebae or in macrophages (Table 3).

The two members of the *Rhabdochlamydiaceae* family, *Rhabdochlamydia crassificans* and *Rhabdochlamydia porcellionis* (Kostanjsek et al., 2004; Corsaro et al., 2007) are, similarly to *Chlamydiaceae,* unable to multiply in amoebae and their eukaryotic host range is still unknown. However, a very recent report by Sixt *et al.* (2013) describes the replication cycle of *R. porcellionis*, a parasite of the crustacean host *Porcellio scaber* in insect cells. A lack of apoptosis induction was observed along the whole replication cycle, a mechanism also described for pathogenic *Chlamydiaceae,* such as *Chlamydia trachomatis* (Ying et al., 2006). *R. crassificans* was first isolated from a cockroach but has also been recently detected by PCR in ticks (*Ixodes ricinus*) collected in Switzerland and in Algeria (Croxatto et al., in press), suggesting that arthropods could act as widespread reservoirs and vectors of transmission for this species. The pathogenicity of *Rhabdochlamydia* has not been extensively studied yet but a few reports suggest a possible role in respiratory infections and in bovine abortion (Haider et al., 2008; Lamoth et al., 2009; Wheelhouse et al., 2010; Lamoth et al., 2011; Niemi et al., 2011).

Up to now, three *Chlamydia*-related bacteria, belonging to three different families, *Waddlia chondrophila*, *Simkania negevensis* and *Estrella lausannensis* have been shown to grow in amoebae and also in several other eukaryotic cells including mammalian macrophages and epithelial cells, fish cells and insect cells (Table 3).

Waddlia chondrophila is an emerging human and animal pathogen, associated with miscarriage in women (Baud et al., 2007; Baud et al., 2011) (Baud and Goy, in press) and abortion in ruminants (Blumer et al., 2011; Barkallah et al., 2013). *Waddlia* might also be implicated in lower respiratory tract infections (Haider et al., 2008; Goy et al., 2009).

These strict intracellular bacteria are able to readily multiply in many different cell types, such as mammalian, fish or insect cells as well as in multiple amoebal species (Michel et al., 2004; Goy et al., 2008; Goy and Greub, 2009; Kebbi-Beghdadi et al., 2011b; Kebbi-Beghdadi et al., 2011a). Replication in amoebae is however less efficient than in macrophages, indicating that protozoa probably do not represent the main reservoir and vector of transmission.

Interestingly, *W. chondrophila* is able to infect endometrial cells *in vitro* and, in this cell line, bacteria stop dividing, enlarge and transform into aberrant bodies (Kebbi-Beghdadi et al., 2011a) (Figure 2A). Since *Waddlia* is associated with adverse pregnancy outcomes in women (Baud et al., 2007; Baud et al., 2011; Baud and Greub, 2011), these aberrant bodies possibly stay hidden in endometrial tissue for many years following a primary asymptomatic or paucisymptomatic infection, and are reactivated by early hormonal changes occurring during the first months of pregnancy, thus leading to a local inflammation that may cause miscarriage. Interestingly, in amoebae, macrophages and epithelial cells, *Waddlia* actively recruits mitochondria around its replicative vacuole immediately at the beginning of bacterial replication (Croxatto and Greub, 2010; Kebbi-Beghdadi et al., 2011a) (Figure 2B). This proximity of mitochondria probably provides the replicating bacteria with a favorable energy and lipid source (Croxatto and Greub, 2010; de Barsy and Greub, 2013).

Similarly to *Waddlia chondrophila* but less rapidly, *Simkania negevensis* can also multiply in amoebae (Kahane et al., 2001; Knab et al., 2011), in insect cells (Sixt et al., 2012) as well as in macrophages and other mammalian cell types (Vero, HEp-2, HeLa), where, similarly to

Chlamydiaceae, it is able to block host cell apoptosis (Kahane et al., 2001; Corsaro and Greub, 2006; Karunakaran et al., 2011). Simkania DNA was amplified from lower respiratory tract samples taken from children with bronchiolitis (Kahane et al., 1998; Greenberg et al., 2003; Friedman et al., 2006) and serological evidence of acute infection was detected in both adults and children suffering from community-acquired pneumonia (Lieberman et al., 1997; Fasoli et al., 2008; Heiskanen-Kosma et al., 2008). However, pathogenicity is still uncertain since seroprevalence is also high in asymptomatic populations (Lieberman et al., 1997; Friedman et al., 2003; Johnsen et al., 2005; Kumar et al., 2005; Friedman et al., 2006). A recent study conducted in Finland on 531 patients with respiratory tract infections did not highlight the presence of this bacteria (Niemi et al., 2011). Since *Simkania negevensis* is often found in mixed infections with viruses and other bacteria, it could also represent an opportunistic microorganism or a predisposing factor rather than a true pathogen.

The third species, *Estrella lausannensis* was isolated from an environmental water sample and is able to grow in numerous amoebal species, including *Dictyostelium discoideum* as well as in fish cells, insect cells, epithelial cells and macrophages although not as efficiently as *Waddlia chondrophila*, (Lienard et al., 2011a, Rusconi et al, submitted, Kebbi-Beghdadi, unpublished data). This broad host range as well as the recovery of *E. lausannensis* DNA in nasopharyngeal swabs of children with pneumonia (Lienard et al., 2011b) suggest a pathogenic potential which deserves further studies.

In conclusion and at least for bacteria of the *Chlamydiales* order, the pathogenicity correlates better with the ability to grow in diverse eukaryotic cells such as epithelial cells, fish cells or insect cells than with the ability to replicate in protozoa and in macrophages. Thus, the paradigm that amoeba-resisting bacteria are likely also resisting to macrophage microbicidal effectors is only partially true. Moreover, our hypothesis that amoebal co-culture will selectively grow virulent bacteria (Greub and Raoult, 2004) should be questioned. This technique should rather be considered as one of the tools used to discover new species, including strict intracellular parasites such as *Chlamydiales* (see below).

Numerous bacteria belonging to the *Chlamydiales* order have been discovered recently from aquatic environments suggesting a much higher diversity among this clade than previously expected (Corsaro et al., 2010a; Corsaro and Work, 2012; Fehr et al., 2013; Steigen et al., 2013; Stride et al., 2013b; Stride et al., 2013a; Corsaro et al., 2013b; Lagkouvardos et al., 2014). Among them are many different species probably causing epitheliocystis in fishes, that have not yet been isolated in culture. Three main reasons may explain why bacteria belonging to the *Chlamydiales* order have long remained largely undetected:

- Commonly used broad-range eubacterial PCRs fail to amplify DNA of *Chlamydiales* bacteria because of the lack of sequence conservation in the 16S rRNA gene (Wilson et al., 1990; Klindworth et al., 2013), (Delafont et al., in press). A real-time quantitative PCR targeting the conserved chlamydial 16S rRNA gene has recently been developed in our group. It can detect 5 DNA copies of any member of the *Chlamydiales* order, exhibits a high specificity for bacteria of this order and do not amplify DNA of any other bacterial clade (Lienard et al., 2011b). This new PCR may fill the gap generally left when using conventional eubacterial PCR.
- Due to particular features of their extracellular membrane (Rusconi et al., 2013), chlamydial elementary bodies are highly resistant to classical bacterial lysis protocols thus hampering DNA extraction (Croxatto et al., 2013). An alternate extraction protocol has thus been set up (Croxatto et al., 2013) that includes a 2 hours proteinase K digestion step ensuring complete bacterial membrane lysis and, consequently, efficient DNA extraction.
- *Chlamydiales* bacteria are obligate intracellular organisms strictly dependent of an eukaryotic host for multiplication. As discussed above, amoebal co-culture is a very good tool to recover these fastidious organisms, but one possible limitation of this approach is the restricted host range observed for some microbial species (Birtles et al., 2000; Horn et al., 2000; Michel et al., 2004; Michel et al., 2005; Coulon et al., 2012; Corsaro et al., 2013b). To circumvent this problem and ensure bacterial recovery in cell culture, different amoebal species (*Acanthamoeba, Naegleria, Hartmanella, Dictyostelium*) as well as

various culture conditions (different temperatures and media) should ideally be tested in parallel (Lienard and Greub, 2011). Moreover, additional cell lines, including insect, fish and mammalian cell lines could also be used.

PCR-based metagenomics offers great possibilities to investigate the diversity of environmental samples (Delafont et al., in press), especially if a pan-*Chlamydiales* PCR is used in conjunction with conventional broad-range eubacterial PCRs or if direct metagenomic is done (without a PCR step). However, metagenomics has one very important limitation: the newly discovered microorganisms are not cultured and strains are not available for further studies.

For this reason, amoebae and more precisely amoebal co-culture is still one of the most useful tools to discover new intracellular bacteria. There is currently no good alternative method to isolate these fastidious microorganisms. However, other cell types, such as insect cells or fish cells should also be used since some species may be restricted to these hosts. Recently, microbial culturomics, ie the use of various culture conditions coupled to MALDI-TOF MS identification was applied to the analysis of the human gut microbiota (Greub, 2012; Lagier et al., 2012) . In a direct comparison, this approach allowed the description of more bacterial species than pyrosequencing and revealed numerous bacteria of low abundance that were undetected in genomic and metagenomic studies. A similar "culturomics" approach could be applied to detect ARMs.

In the future, development of automatized inoculation and incubation systems will improve the feasibility of high throughput culturomics approaches (including cell-culture based culturomics), thus helping to isolate new amoebae-resisting microorganisms.

7. Conclusions

The ecology of amoeba-resisting bacteria such as *Parachlamydiaceae*, *Legionella* and *Mycobacteria* has major public health implications, since amoebae may serve as a widespread reservoir and important mode of transmission for these bacteria. Moreover, the evolution of these bacteria has likely been modulated by their intra-amoebal life where they

may have developed virulence traits. However, amoeba-resisting bacteria do not necessarily grow in macrophages and do not necessarily represent mammalian pathogens. Thus, amoebal co-culture only represents one tool to recover fastidious intracellular bacteria from complex microbiota, but should be coupled to a wide array of cell-culture based approaches to unravel the yet largely uncovered biodiversity of intracellular bacteria.

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1 **Figure legends**

Table 1.

Multiple roles played by amoebae in the ecology of their ARMs. Adapted from (Greub, 2009).

Table 2.

Advantages and limitations of two methods using amoebae as tool to discover new ARMs.

Table 3.

Ability of harmless or pathogenic *Chlamydiales* to grow in diverse host cells.
Please refer to text for references supporting data provided in this table.
*induced apoptosis is limiting *P. acanthamoebae* replication

Figure 1.

Schematic illustration of amoebal co-culture and amoebal enrichment, two efficient methods used to isolate intracellular microorganisms. Adapted from (Lienard and Greub, 2011).

Figure 2.

A: *Waddlia chondrophila* in human endometrial cells 5 days post infection. Bacteria (green) are stained with a polyclonal rabbit anti-*Waddlia* antibody and endometrial cells (red) are stained with Texas Red-conjugated Concanavalin A. Please note that aberrant bodies (AB) are much larger than reticulate bodies (RB).
B: Electron micrograph of *A. castellanii* infected by *W. chondrophila*. EB: elementary body; RB: reticulate body; M: mitochondria; n: nucleus.

Tables and Figures

Table 1

Role of amoebae	Selected references
Replicative niche	(Swanson and Isberg, 1995; Brieland et al., 1997; Greub and Raoult, 2004; Dusserre et al., 2008)
Widespread reservoir	(Thomas et al., 2006b; Pagnier et al., 2008; Thomas et al., 2008; Corsaro et al., 2009; Loret and Greub, 2010)
Protective armour	(Steinert et al., 1998; Greub and Raoult, 2003a; Coulon et al., 2010; Loret and Greub, 2010)
Mode of dissemination	(Berk et al., 1998; Marolda et al., 1999; Greub and Raoult, 2002b; Abd et al., 2003)
Trojan horse	(Cirillo et al., 1997; Winiecka-Krusnell and Linder, 1999; Greub and Raoult, 2002, 2004)
Gene exchange	(Boyer et al., 2009; Greub, 2009; Saisongkorh et al., 2010; Lamrabet et al., 2012)
Selection of virulence traits	(Cirillo et al., 1997; Steenbergen et al., 2001; Molmeret et al., 2005; Goy et al., 2007)
Adaptation to macrophages	(Gao et al., 1997; Goy et al., 2008; Salah et al., 2009; Escoll et al., 2013)

Table 2

Amoebal co-culture	Amoebal enrichment
Advantages	**Limitations**
Allows large scale screening (in 24 to 48 wells microplates)	Fastidious, time consuming, not easily scalable
Provides a bacterial species or a virus in pure culture (considering the amoebae as a cell background)	Provides amoebae possibly containing the ARMs; need of sub-culture (co-culture, mammalian cell culture) to isolate ARMs
May be performed, in parallel wells, in presence and in absence of different antibiotics and antifungals	Prone to contamination by fungi (amphotericin precluded, given its negative impact on amoebae)
Various screening approaches, such as Gimenez stain, PCR, etc, may be applied	Screening mainly by microscopy
Limitations	**Advantages**
No knowledge of the corresponding amoebal host	Allows identification of both the ARM and its natural amoebal host
Recovers only ARMs able to grow within the amoebal species used for the co-culture (often *Acanthamoeba*)	Allows identification of ARM independently of their amoebal host range (for example, *Neochlamydia hartmannellae* grow only in *Hartmanella vermiformis* and would not have been detected by *Acanthamoeba* based amoebal co-culture)

Table 3

Family-level lineage	Species	Replication in amoebae	Replication in insect cells	Replication in fish cells	Replication in macrophages	Replication in epithelial cells	Pathogenicity	Host spectrum
Chlamydiaceae	*C. pneumoniae*	-	unknown	unknown	+/-	+	+	cold and warm blooded vertebrates
	C. abortus	-	unknown	unknown	+/-	+	+	cold and warm blooded vertebrates
	C. trachomatis	-	+/-	unknown	+/-	+	+	humans
Rhabdochlamydiaceae	*R. crassificans*	-	unknown	unknown	unknown	unknown	+/-	insects humans? bovines?
	R. porcellionis	-	+	unknown	unknown	unknown	unknown	crustaceans humans? bovines?
Waddliaceae	*W. chondrophila*	+	+	+	+	+	+	warm blooded vertebrates
Simkaniaceae	*S. negevensis*	+	+	unknown	+	+	+/-	humans
Criblamydiaceae	*E. lausannensis*	+	+	+	+	+	unknown	unknown
	C. sequanensis	+	unknown	unknown	unknown	unknown	unknown	unknown
Parachlamydiaceae	*P. acanthamoebae*	+	-	+/-	+/-*	+/-	+	bovines
	P. amoebophila	+	-	unknown	unknown	-	-	protozoa
	P. naegleriophila	+	unknown	unknown	unknown	unknown	unknown	humans protozoa
	N. hartmanellae	+	unknown	unknown	unknown	unknown	-	protozoa

Figure 1

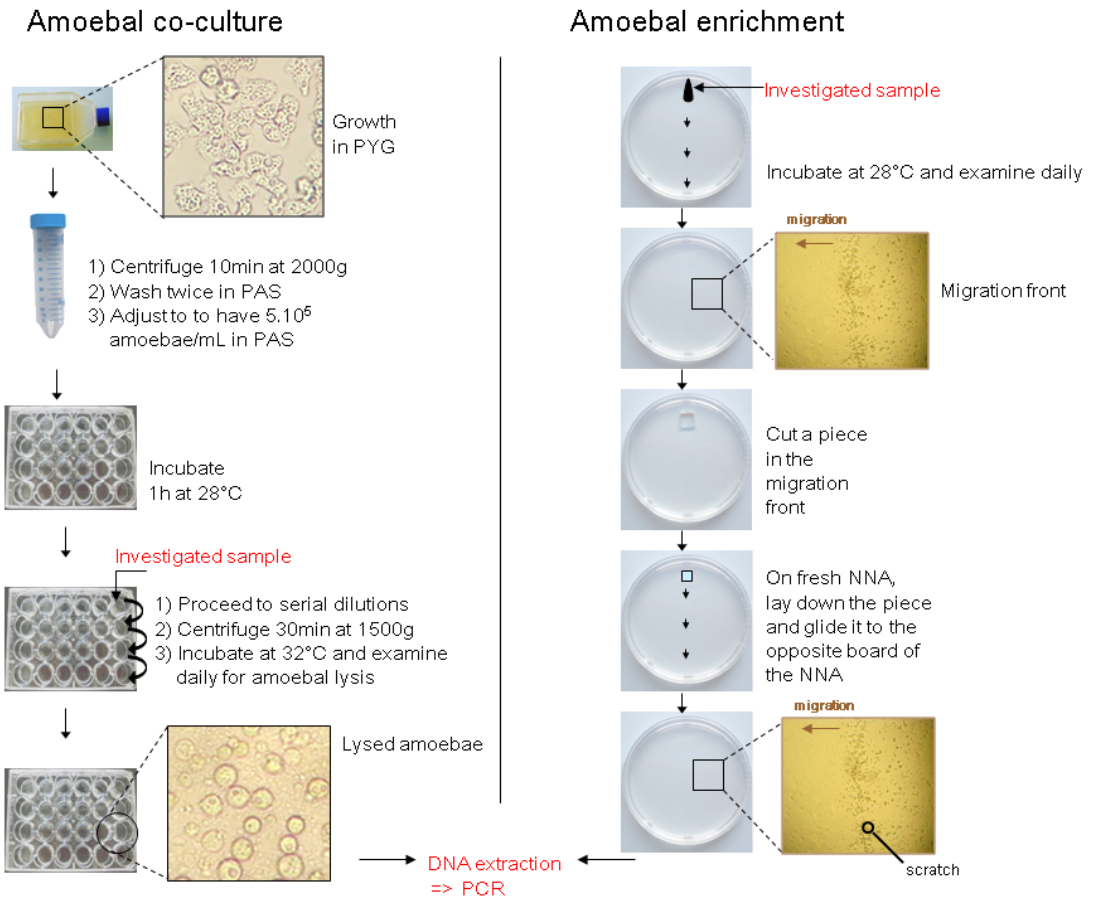
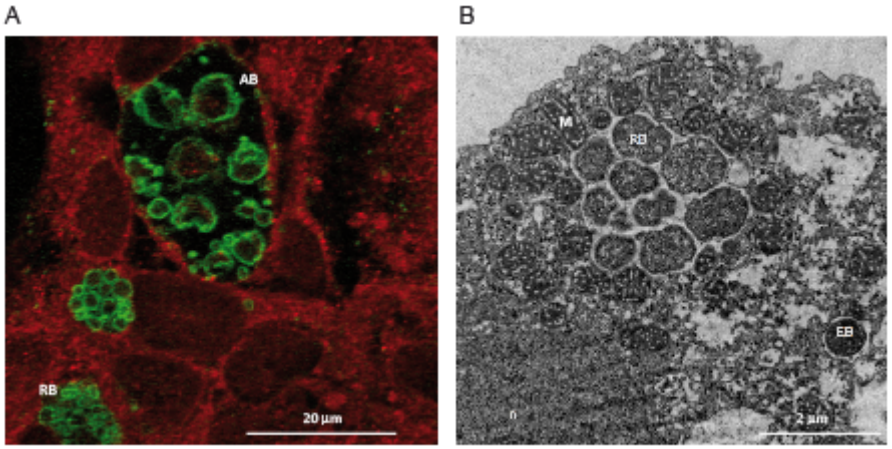
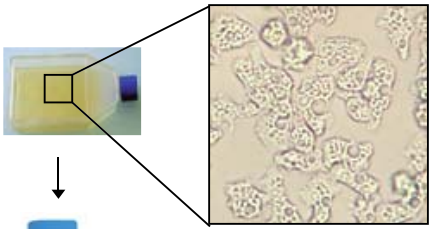


Figure 2



Amoebal co-culture

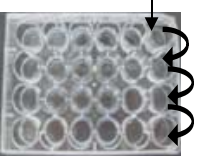


Growth
in PYG

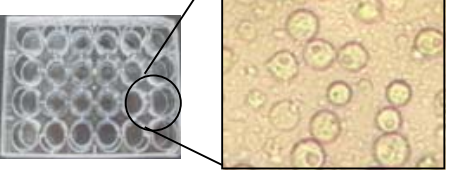
1) Centrifuge 10min at 2000g
2) Wash twice in PAS
3) Adjust to to have 5.10^5 amoebae/mL in PAS

Incubate
1h at 28°C

Investigated sample



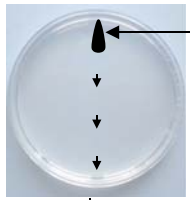
1) Proceed to serial dilutions
2) Centrifuge 30min at 1500g
3) Incubate at 32°C and examine daily for amoebal lysis



Lysed amoebae

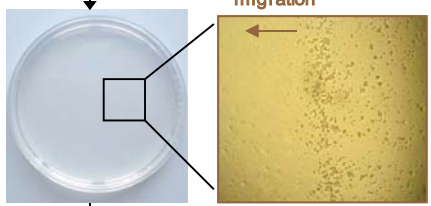
DNA extraction
=> PCR

Amoebal enrichment



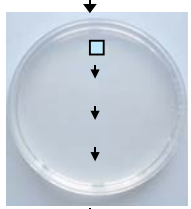
Investigated sample

Incubate at 28°C and examine daily

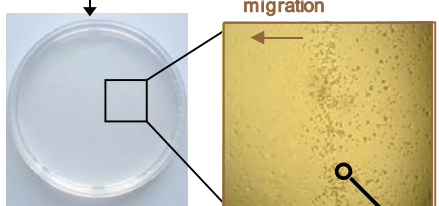


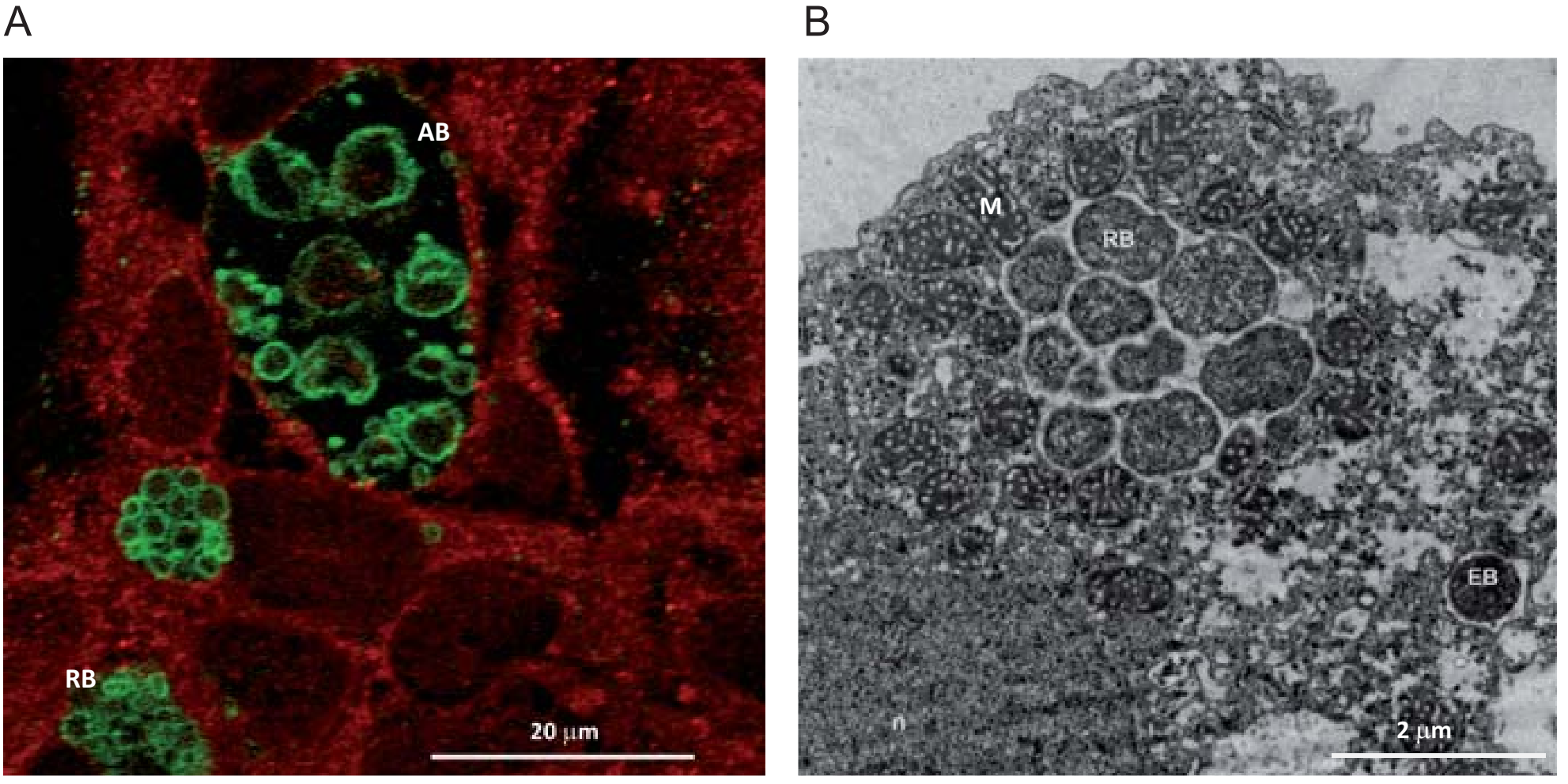
Migration front

Cut a piece
in the
migration
front



On fresh NNA,
lay down the piece
and glide it to the
opposite board of
the NNA




A
AB
RB
20 μm
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n
2 μm