REVIEW ARTICLE

Amoebal pathogens as emerging causal agents of pneumonia

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Abstract

Despite using modern microbiological diagnostic approaches, the aetiological agents of pneumonia remain unidentified in about 50% of cases. Some bacteria that grow poorly or not at all in axenic media used in routine clinical bacteriology laboratory but which can develop inside amoebae may be the agents of these lower respiratory tract infections (RTIs) of unexplained aetiology. Such amoebae-resisting bacteria, which coevolved with amoebae to resist their microbicidal machinery, may have developed virulence traits that help them survive within human macrophages, i.e. the first line of innate immune defence in the lung. We review here the current evidence for the emerging pathogenic role of various amoebae-resisting microorganisms as agents of RTIs in humans. Specifically, we discuss the emerging pathogenic roles of *Legionella*-like amoebal pathogens, novel *Chlamydiae* (*Parachlamydia acanthamoebae*, *Simkania negevensis*), waterborne mycobacteria and *Bradyrhizobiaceae* (*Bosea* and *Afipia* spp.).

Introduction

Despite advances in antibiotic therapy, pneumonia remains one of the leading infectious causes of death in developed countries and a major cause of morbidity, especially in the elderly population and among patients with chronic underlying diseases (Mandell *et al.*, 2007). Although a broad spectrum of microbial pathogens have been recognized as causal agents of respiratory tract infections (RTIs), the offending microorganism remains unknown in about half of the cases of community-acquired pneumonia (CAP) (Bochud *et al.*, 2001; Echols *et al.*, 2008), and three quarters of the cases of nosocomial pneumonia (Costa *et al.*, 2001).

Microorganisms causing pneumonia may be acquired from respiratory droplets through human-to-human contact or from aerosolized particles from an animal or environmental reservoir. The epidemiology of RTI thus strongly depends on the interactions between humans and their ecosystem and evolves according to environmental changes due to human activities, climatic or ecological perturbations. Pandemics of influenza or the emergence of new respiratory diseases such as severe acute respiratory syndrome are dramatic illustrations of these phenomena. To some extent, agents of pneumonia have been identified in the context of outbreaks or case series occurring in a particular setting. For instance, psittacosis (parrot fever) was described for the first time in seven individuals exposed to pet birds in Switzerland in the 19th century (Ritter, 1880). More recently, the epidemics of severe pneumonia affecting war veterans in a hotel in Philadelphia in 1976 led to the discovery of the fastidious gram-negative rod *Legionella pneumophila* as a causal agent of respiratory diseases (Fraser *et al.*, 1977; McDade *et al.*, 1977). In this latter situation, water was found to be the source of contamination. Rowbotham (1980, 1983) then demonstrated that *L. pneumophila* may multiply within free-living amoebae and hypothesized that these protists may represent a reservoir for these intracellular bacteria.

Free-living amoebae live in water, soil and at the water–air interface. As they generally use bacteria as their main nutritional source, they are especially present in large quantities in sediments and biofilms (Rodriguez-Zaragoza, 1994). Thus, humans have been increasingly exposed to amoebae and to their related bacterial pathogens with the progressive development of various modern man-made water systems such as water-treatment plants, cooling

towers, air conditioners, humidifiers, spas and swimming pools (Rodriguez-Zaragoza, 1994; Greub & Raoult, 2004; Pagnier *et al.*, 2009a). Apart from *L. pneumophila*, which has mainly been recognized due to the dramatic importance of the Philadelphia outbreak, many other bacteria that resist the phagocytic amoebae may also use these protists as widespread reservoirs and may have acquired virulence traits promoting their resistance to macrophages. Interestingly, some of these amoebae-resisting bacteria have also been discovered during outbreaks of RTI (Herwaldt *et al.*, 1984; Birtles *et al.*, 1997). Indeed, given their intracellular lifestyle, they either do not grow or only poorly grow in conventional axenic media. It is important that the microbiology community be well aware of these new emerging human agents of pneumonia and develop new diagnostic tools for their identification. Amoebal coculture and amoebal enrichment coupled with detection of potential intra-amoebal bacteria have been demonstrated to be largely successful in identifying a large biodiversity of new pathogens from patients (Greub *et al.*, 2004b; Thomas *et al.*, 2006; Corsaro *et al.*, 2009; Pagnier *et al.*, 2009a, b).

In this review, we intend to present the current evidence of the role of various amoebae-resisting bacteria as agents of RTI in humans. More specifically, we will discuss the likely role of *Legionella*-like amoebal pathogens (LLAPs), novel *Chlamydiae* (*Parachlamydia acanthamoebae*, *Simkania negevensis*), waterborne mycobacteria and *Bradyrhizobiaceae* (*Bosea* and *Afipia* spp.).

Diagnostic tools for the identification of amoebae-resisting bacteria

For a better characterization of the microbial biodiversity, various molecular approaches are available, including a coupled cloning and sequencing approach, and metagenomics using new pyrosequencing techniques such as the 454 and Solexa/Illumina technologies. However, such sequence-based ecological studies do not provide the strains for subsequent studies, and consequently culture-based ecological studies are of equal if not greater importance. However, most culture-based studies are biased towards bacteria able to grow efficiently on different axenic media, broths and/or agar plates, and approaches that selectively amplify amoebae-resisting bacteria may be useful. Two main approaches, amoebal coculture and amoebal enrichment, have been applied so far to recover amoebae-resisting bacteria in culture (Fig. 1). Amoebal coculture is a cell culture method in which axenic amoebae are used as a host cell culture, whereas amoebal enrichment uses an enteric bacterium such as *Escherichia coli* as a food source for amoebae that are potentially present in the investigated sample. Once isolated by amoebal enrichment, the amoebae may then be studied for the possible presence of intra-amoebal microorganisms. Both approaches have been successfully used in recent years to uncover a variety of amoebae-resisting microorganisms from both environmental and clinical samples (Adekambi *et al.*, 2004; Greub *et al.*, 2004b; Thomas *et al.*, 2006, 2008; Loret *et al.*, 2008; Corsaro *et al.*, 2009; Pagnier *et al.*, 2009b).

Understanding the pathogenic role of amoebae-resisting bacteria: a comprehensive model approach

The availability of a bacterial strain growing in amoebae allows its pathogenic potential to be tested, for instance as an agent of pneumonia, using the comprehensive approach that has been applied to *P. acanthamoebae* (Greub, 2009). This global strategy includes (1) testing the permissiveness of lung fibroblasts, pneumocytes and alveolar macrophages to the new bacterial species; (2) developing diagnostic tools (serology, antigen-detection assays, PCR and immunohistochemistry) to study patients with and without lower RTIs; and (3) investigating the pathogenic role of some selected species in an animal model of pneumonia. For species that emerge as new pathogens or exhibit very peculiar interesting biological phenotypes, the availability of a given strain also allows better understanding of the biology of the species involved, using functional genomics, proteomics and cell biology.

Amoebal pathogens as causal agents of pneumonia: clinical evidence

Legionella species

Legionella pneumophila

The potential role of water systems as a reservoir of human respiratory diseases was recognized for the first time when *L. pneumophila* was identified as the causal agent of an outbreak of pneumonia in Philadelphia in 1976 (Fraser *et al.*, 1977; McDade *et al.*, 1977). This bacterium is widespread in our aquatic environment including man-made water systems and is one of the first examples of amoebae-resisting bacteria that has been described (Rowbotham, 1980, 1983). It is estimated to account for 2–7% of all cases of CAP affecting both immunocompromised and immunocompetent hosts (Doebbeling & Wenzel, 1987; Marrie *et al.*, 1989; Fang *et al.*, 1990a; Woodhead, 2002). Nosocomial outbreaks of Legionnaires' disease are also frequently reported and may result from bronchoaspiration of contaminated potable water rather than inhalation of aerosolized particules (Blatt *et al.*, 1993; Sabria & Yu, 2002). In addition to potentially severe respiratory diseases, legionellosis may present as a flu-like syndrome called pontiac fever, which may be

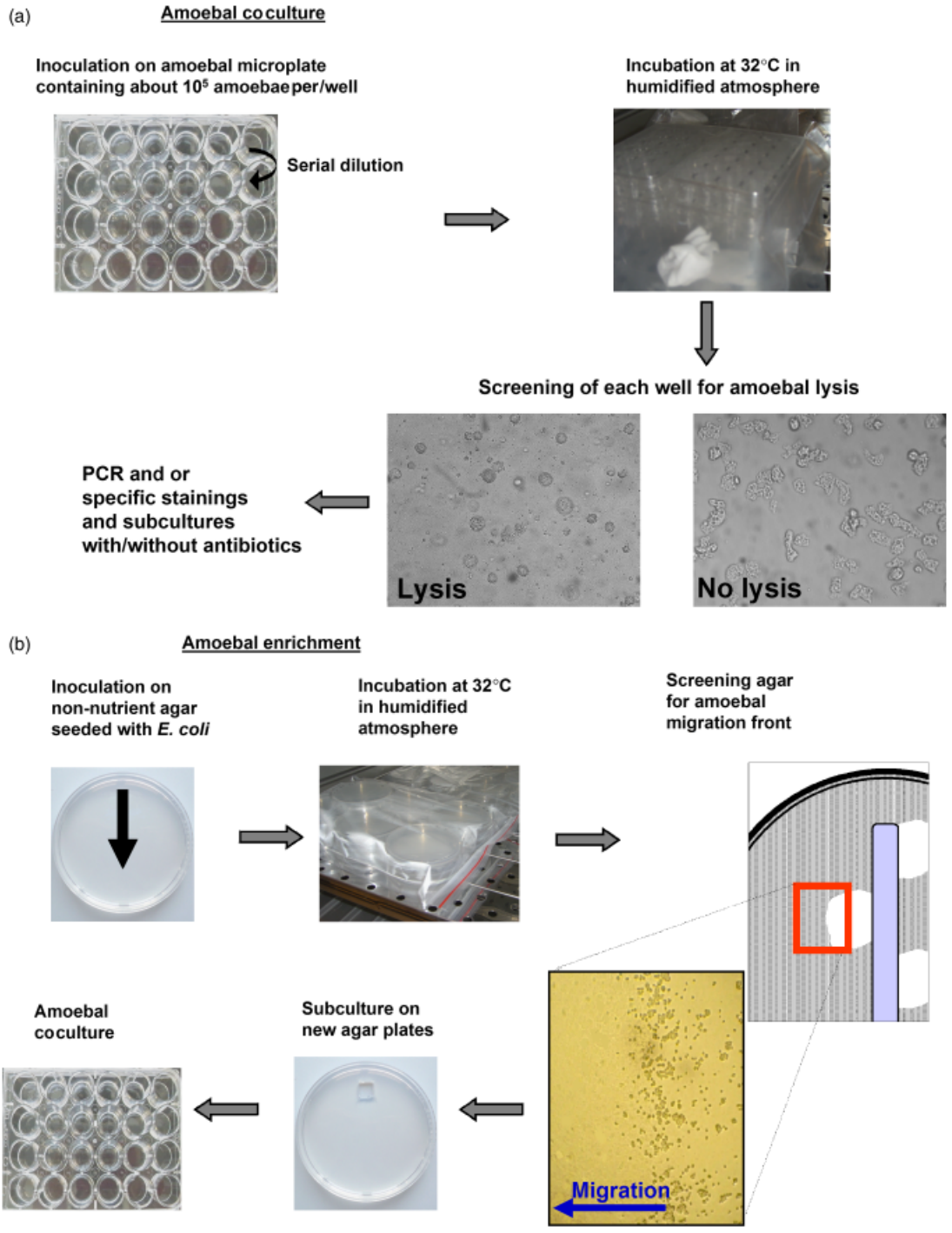

Fig. 1. Culture-based approaches that may be used to selectively grow amoebae-resisting microorganisms. (a) Amoebal coculture is a cell culture approach that uses amoebae as cell background and that can be used to isolate strict intracellular bacteria starting directly from clinical and/or environmental samples or by inoculation of free-living amoebae potentially containing amoebal pathogens or endosymbionts. Screening may then be achieved with various stainings and/or by PCRs. Subcultures on fresh amoebae in presence/absence of different antibiotics may help in isolating a given strain from heavily contaminated environmental samples. (b) Free-living amoebae may be isolated by amoebal enrichment, which consists in the inoculation of clinical and/or environmental samples on non-nutritive agar plates previously seeded with *Escherichia coli* and/or *Enterobacter cloacae*, which serve as a food source for the amoebae potentially present in the investigated sample. Once inoculated, agar plates can be screened daily for the presence of an amoebal migration front and, when positive, subcultured on new non-nutritive agar. Amoebae can then be screened for the presence of amoebae-resisting microorganisms by amoebal coculture and or molecular approaches.

misdiagnosed as a viral infection and whose incidence is possibly underestimated (Doebbeling & Wenzel, 1987).

Legionella pneumophila is a fastidious gram-negative rod which is rarely detected by examination of gram stains of clinical samples and which needs buffered charcoal yeast extract (BCYE) agar to be grown. The availability of a urinary-specific antigen test for the detection of *L. pneumophila* serogroup 1, which accounts for about 85–90% of legionellosis in Europe and America, allows a rapid diagnosis of the disease. This test, in combination with cultures, exhibits an overall good sensitivity and specificity and its widespread use has improved the identification of *Legionella* pneumonia in hospitalized patients (Waterer *et al*., 2001). Molecular diagnostic tools have shown promising results but these methods lack standardization and their availability is limited (Waterer *et al*., 2001; Murdoch, 2003). The actual role of *L. pneumophila* in less severe pneumonia in the community as well as the incidence of respiratory diseases attributed to other *Legionella* species thus remain difficult to estimate (Waterer *et al*., 2001; Murdoch, 2003).

Legionella species other than *L. pneumophila*

Since the discovery of *L. pneumophila*, about 45 other *Legionella* species have been identified (Benson & Fields, 1998). These species share the same aquatic environment as *L. pneumophila* and about half of them have been associated with respiratory infections in humans (Table 1) (Muder & Yu, 2002; Roig *et al*., 2003). However, only a limited number of species seems to be relevant pathogens, whereas the others have been identified as the cause of pneumonia in anecdotal case reports. In a multinational survey, they have been estimated to account for 5–10% of legionellosis (Benin *et al*., 2002; Yu *et al*., 2002). The distribution of *Legionella* spp. is variable around the world: for instance, *Legionella longbeachae* is responsible for as many as 30% cases of *Legionella* diseases in Australia and New Zealand, whereas it accounts for only 3–4% of cases on the European and American continents (Yu *et al*., 2002). *Legionella bozemanii*, *Legionella micdadei*, *Legionella dumoffii*, *Legionella anisa* and *Legionella feelei* may account for most of the 1–5% remaining cases of Legionnaires' diseases (Fang *et al*., 1989; McNally *et al*., 2000; Benin *et al*., 2002; Muder & Yu, 2002; Yu *et al*., 2002). In contrast to *L. pneumophila* infections, pulmonary diseases attributed to other *Legionella* spp. have been mainly reported in a nosocomial context affecting immunocompromised patients such as haematopoietic or solid organ transplant recipients, patients under long-term corticoid therapy or splenectomized patients (Muder *et al*., 1983; Fang *et al*., 1989; Muder & Yu, 2002). With the exception of *L. longbeachae*, other *Legionella* spp. are rarely involved in CAP, although community outbreaks of pontiac fever have also been reported with *L. anisa*, *L. micdadei*, *L. feelei* and *Legionella sainthelensi* (Herwaldt *et al*., 1984; Goldberg *et al*., 1989; Fenstersheib *et al*., 1990; Loeb *et al*., 1999).

Most *Legionella* species grow on BCYE agar media, but specific cultures for *Legionella* are not routinely performed in cases of CAP. Moreover, these media usually contain antibiotics for the selection of *L. pneumophila* that may inhibit the growth of some other *Legionella* species. The urinary antigen test does not detect species other than *L. pneumophila* serogroup 1, and PCR methods are as yet not widely used. For these reasons, *Legionella* spp. other than *L. pneumophila* are rarely identified as causal agents of infections and their role in the epidemiology of community- and hospital-acquired pneumonia has not been assessed precisely. The fact that these species exhibit a variable ability to infect and proliferate within amoebae may partly explain why they are less frequently involved in RTIs compared with *L. pneumophila* (Neumeister *et al*., 1997; Gao *et al*., 1999).

LLAPs

Historically, the term *Legionella*-like amoebal pathogens was introduced to designate obligate intracellular parasites of free-living amoebae which were closely related to the legionellae (Fig. 2) and which, unlike other *Legionella* spp., exhibited little or no growth on conventional bacteriological media such as BCYE agar (Rowbotham, 1986; Greub & Raoult, 2004). Most strains were originally isolated from water supplies during investigations of individual cases or outbreaks of Legionnaires' disease (Adeleke *et al*., 1996; Birtles *et al*., 1996). Because of their limited ability to grow in culture, these bacteria could not be completely characterized and were initially designated by numbers (e.g. LLAP-1–14). The first isolation of an LLAP (LLAP-3) in a clinical specimen was reported in 1991 using amoebal enrichment of the sputum of a patient with pneumonia who exhibited seroconversion against this strain (Fry *et al*., 1991). Phylogenetic analyses subsequently allowed its classification in the species *Legionella lytica* (Birtles *et al*., 1996), whereas other LLAP strains were characterized and assigned to new species of *Legionella* (*Legionella drozanskii*, *Legionella rowbothamii*, *Legionella fallonii*, *Legionella drancourtii*) (Adeleke *et al*., 1996, 2001; La Scola *et al*., 2004). The term of LLAPs thus has been retained for historical reasons, as most of these species have now been recognized to belong phylogenetically to the *Legionella* genus. Moreover, most of them are currently able to grow on BCYE agar because of the improvement in the quality of media and possibly because of a progressive adaptation by successive subcultures on amoebae.

The pathogenic role of LLAPs has been investigated in two series of patients suggesting that these fastidious bacteria may be a cause of pneumonia in some cases (Table 1) (McNally *et al*., 2000; Marrie *et al*., 2001). A French study

Table 1. Pathogenic role of *Legionella* spp. in pneumonia

Legionella species	Pathogenic role in pneumonia	References
L. pneumophila	2–7% of community-acquired pneumonia (8% of those requiring ICU), 5% of nosocomial pneumonia	Doebbeling & Wenzel (1987), Marrie *et al*. (1989), Fang *et al*. (1990a), Woodhead (2002)
	Most frequent cause of *Legionella* pneumonia (91.5%)	Yu *et al*. (2002)
L. longbeachae	Second cause of *Legionella* pneumonia worldwide (3.9%)	Yu *et al*. (2002)
	Frequent cause of potentially severe community-acquired pneumonia in Australia, New Zealand and South East Asia	Grove *et al*. (2002), Yu *et al*. (2002), Phares *et al*. (2007)
	Few cases reported in Europe or United States	McKinney *et al*. (1981), Yu *et al*. (2002), McClelland *et al*. (2004), Kumpers *et al*. (2008)
L. bozemanii	Third cause of community-acquired *Legionella* pneumonia (2.4%)	Fang *et al*. (1989), McNally *et al*. (2000), Yu *et al*. (2002)
	Cause of severe pneumonia in immunocompromised patients (frequent complications: empyema, cavitation)	Fang *et al*. (1989), Swinburn *et al*. (1988), Taylor & Albrecht (1995), Harris *et al*. (1998), Muder & Yu (2002)
L. micdadei	Cause of life-threatening pneumonia in immunocompromised patients	Myerowitz *et al*. (1979), Muder *et al*. (1983), Fang *et al*. (1987), Doebbeling *et al*. (1989), Muder & Yu (2002)
	Purulent pneumonia or pulmonary abscesses in solid-organ transplant recipients	Myerowitz *et al*. (1979), Rogers *et al*. (1979), Mehta *et al*. (1983), Ernst *et al*. (1998), Knirsch *et al*. (2000)
L. anisa	Rare cause of community-acquired or nosocomial pneumonia	McNally *et al*. (2000), Yu *et al*. (2002), La Scola *et al*. (2003b), Doleans *et al*. (2004)
	Identified as the cause of an outbreak of pontiac fever in California	Fenstersheib *et al*. (1990)
L dumoffii	Some cases of pneumonia reported mainly in immunocompromised patients	Fang *et al*. (1990b), Murdoch & Chambers (2000), Muder & Yu (2002), Yu *et al*. (2002)
L. feeleii	About 10 cases of pneumonia reported in the literature (75% in immunocompromised patients)	Lee *et al*. (2009)
	One outbreak of pontiac fever in an automobile plant	Herwaldt *et al*. (1984)
L. jordanis	Subacute or chronic respiratory infection with constitutional symptoms (rare cases described)	Thacker *et al*. (1988b), Vinh *et al*. (2007)
L. sainthelensi	Two outbreaks of respiratory infections in nursing homes (Canada)	Loeb *et al*. (1999)
L. maceachernii	Case reports of pneumonia in immunocompromised patients	Wilkinson *et al*. (1985a), Thomas *et al*. (1992), Dumoff *et al*. (2004), van Dam *et al*. (2006)
L. gormanii	Case reports of pneumonia in immunocompromised patients	Griffith *et al*. (1988), Ephros *et al*. (1989), Towns *et al*. (1994)
L. wadsworthii	Case reports of pneumonia in immunocompromised patients	Edelstein *et al*. (1982), Yu *et al*. (2002)
L. cincinnatiensis	Case reports of pneumonia in renal transplant recipients or haemodialysis patient	Thacker *et al*. (1988a), Jernigan *et al*. (1994)
L. tucsonensis	Case reports of pneumonia in immunocompromised patients	Thacker *et al*. (1989), Doleans *et al*. (2004)
L. oakridgensis	3 cases of pneumonia observed in patients with connective tissue diseases	Tang *et al*. (1985), Lo Presti *et al*. (2000)
L. parisiensis	One case report of pneumonia in a liver transplant recipient	Lo Presti *et al*. (1997)
L. lansingensis	One case report of pneumonia in a patient with chronic lymphocytic leukaemia	Thacker *et al*. (1992)
L. hackeliae	One case report of pneumonia immunocompromised patient	Wilkinson *et al*. (1985b)
L. lytica (LLAP 3, 7 and 9)	Seroconversion observed in cases of community-acquired pneumonia	Fry *et al*. (1991), McNally *et al*. (2000)
L. drancourtii (LLAP 4 and 12)	Seroprevalence study suggesting a pathogenic role	Marrie *et al*. (2001)
L. fallonii (LLAP 10)	Seroprevalence study suggesting a pathogenic role	McNally *et al*. (2000)
L. rowbothamii (LLAP 6)	Seroprevalence study suggesting a pathogenic role	McNally *et al*. (2000)
L. drozanskii (LLAP 1)	Seroprevalence study suggesting a pathogenic role	McNally *et al*. (2000), Marrie *et al*. (2001)

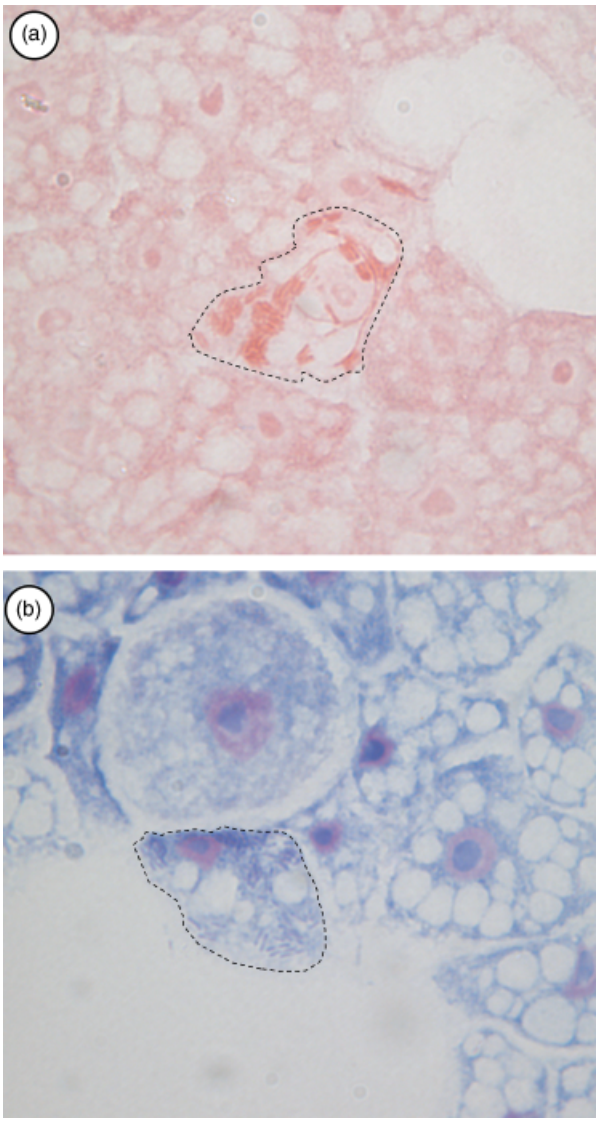

Fig. 2. *Legionella drancourtii*, i.e. LLAP-12, within *Acanthamoeba castellannii* as seen by gram staining (a) and Diff-Quick staining (modified May–Grünwald Giemsa) (b), respectively. Infected amoebae, which contain numerous rod-shaped bacteria, are circled by a dotted line. Microscopy performed 24 h postinfection at a low MOI of about 1/10; × 1000 magnification.

reported serological evidence for recent infections with LLAP in 1.4% of CAP (Marrie *et al.*, 2001). Most of them were attributed to LLAP-4 (*L. drancourtii*). An alternative potential pathogen was, however, isolated in about half of these cases. Similarly, a significant rise in antibodies against LLAP-1, -3, -6, -9 and -10 was reported in 7% of patients with CAP of unknown aetiology in a North American series (McNally *et al.*, 2000). Data from other parts of the world or other subsets of population are lacking, although the ubiquity and biodiversity of *Legionella* spp., including LLAPs, in water systems has been reported worldwide (Thomas *et al.*, 2006; Diederen *et al.*, 2007; Wery *et al.*, 2008; Hsu *et al.*, 2009). The role of LLAPs in human diseases is difficult to assess, as their pathogenic role was suggested only by serological tests in relatively few cases, and in the absence of direct microbiological documentation by other diagnostic tools such as culture or molecular methods. Moreover, *in vitro* studies or animal models supporting their pathogenicity are currently lacking.

Chlamydia-related bacteria

Molecular and phylogenetic studies have recently revealed a rich diversity of microorganisms within the order *Chlamydiales* and allowed the identification of new families distinct from the well-known *Chlamydiaceae* (Everett *et al.*, 1999; Corsaro & Greub, 2006; Greub, 2009). The term '*Chlamydia*-like organisms', 'novel *Chlamydiae*', '*Chlamydia*-related bacteria' or 'amoebae-resistant *Chlamydiae*' have been used to designate these strict intracellular bacteria that may infect and survive within free-living amoebae. Growing evidence suggests that some of them may be the cause of RTIs in humans (Friedman *et al.*, 2003; Corsaro & Greub, 2006; Greub, 2009).

Simkaniaceae

Simkania negevensis, formerly called microorganism 'Z' or 'Simkania Z', is the member of these *Chlamydia*-like organisms whose implication in RTIs has been most extensively studied. This bacterium may easily grow within the *Acanthamoeba* amoeba (Fig. 3) and may also use free-living amoebae as a widespread environmental reservoir. *Simkania negevensis* was first described in 1993 as a cell culture contaminant of unknown origin exhibiting a typical two-stage developmental cycle, with infectious elementary bodies and replicative reticulate bodies (Fig. 4), but differing significantly from *Chlamydiaceae* (Kahane *et al.*, 1993). Its seroprevalence in the population displays important variations around the world, having been reported to be as high as 55–80% in Israel and only 4% in Japan (Friedman *et al.*, 1999, 2006; Johnsen *et al.*, 2005; Yamaguchi *et al.*, 2005). These differences may be partially due to the respective sensitivity and specificity of the serological approaches used and to the cut-off defining positivity. The involvement of *S. negevensis* in RTIs has been investigated in several large cohorts of patients in Europe, the Middle East and America using serological or molecular diagnostic methods (Table 2) (Kahane *et al.*, 1998; Lieberman *et al.*, 2002, 1997; Greenberg *et al.*, 2003; Kumar *et al.*, 2005; Friedman *et al.*, 2006; Fasoli *et al.*, 2008; Heiskanen-Kosma *et al.*, 2008; Nascimento-Carvalho *et al.*, 2009). Some of these analyses reported an association with CAP, exacerbations of chronic obstructive

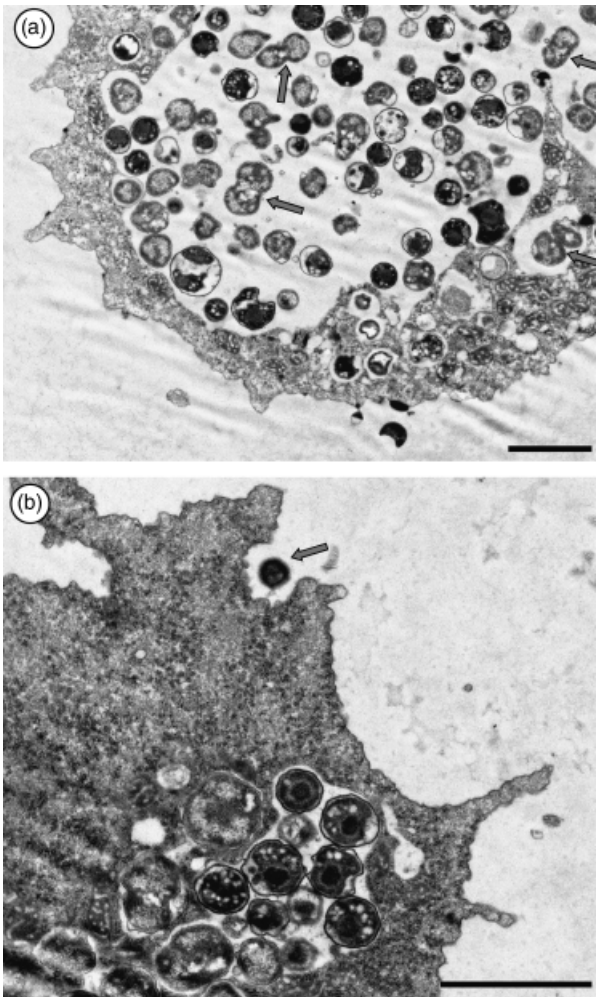

Fig. 3. *Simkania negevensis* within *Acanthamoeba castellannii*, as seen by electron microscopy 24 h postinfection. Please note (a) the presence of several dividing reticulate bodies (arrows). (b) An elementary body in the process of being phagocytized (arrow). Magnification × 7000 and × 10 000, respectively. Scale bar = 2 μm.

pulmonary diseases or bronchiolitis in adults and children (Kahane *et al.*, 1998; Lieberman *et al.*, 2002, 1997; Friedman *et al.*, 2003, 2006; Greenberg *et al.*, 2003; Fasoli *et al.*, 2008; Heiskanen-Kosma *et al.*, 2008; Nascimento-Carvalho *et al.*, 2009). The importance of this bacterium as a causal agent of CAP is, however, difficult to evaluate and seems relatively marginal (< 2% of all aetiologies) in view of these results. Its incidence may be higher in some populations or ethnic groups where a high seroprevalence of *S. negevensis* has been documented, for example in the Middle East (Bedouins) (Kahane *et al.*, 1998; Friedman *et al.*, 1999) or Northern Canada (Inuits) (Greenberg *et al.*, 2003), whereas it remains to be determined in many other parts of the world. A study carried out in Brooklyn (NY) deserves mention as it did not identify any association with respiratory diseases despite a

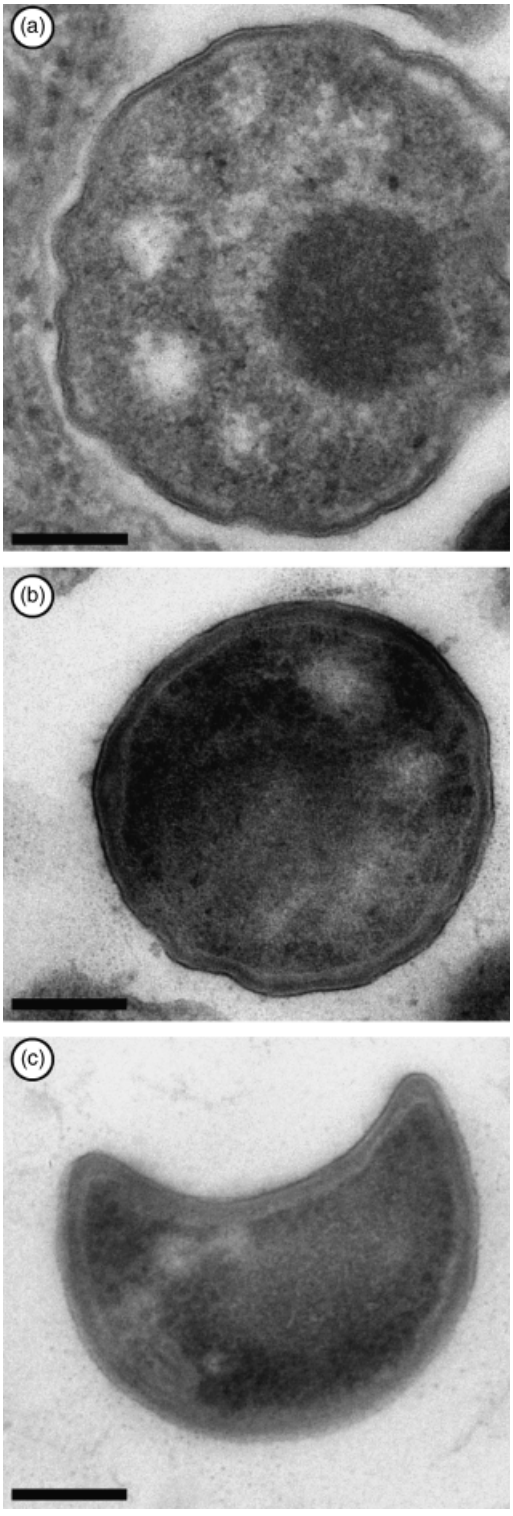

Fig. 4. The two developmental stages of *Simkania negevensis*: the reticulate body, i.e. the metabolically active dividing stage (a) and the elementary body, i.e. the infectious stage (b). *Simkania negevensis* may also infrequently exhibit a third stage, the crescent body (initially reported for *Parachlamydia acanthamoebae*), which may be seen on some electron microscopy preparations (c). Electron micrographs: magnification × 70 000; Scale bar = 0.2 μm.

Table 2. *Simkania negevensis* as causal agents of pneumonia: review of the literature

Population (country) and number of patients	Disease	Diagnostic method	Positive results	*P* value (if controls)	Recent infection* and no alternative pathogen	References
Adults (Israel) 308	CAP	Serology (IgG, IgA)	112 (37%)	No control	4 (1.3%)	Lieberman *et al*. (1997)
Infants (Israel) 239	Bronchiolitis	Culture and/or PCR (NP swabs)	60 (25%)	$P < 0.001$	38 (16%)	Kahane *et al*. (1998)
Adults (Israel) 190	COPD exacerbation	Serology (IgG, IgA)	120 (63%)	Not significant	1 (0.5%)	Lieberman *et al*. (2002)
Infants (Canada) 22	Bronchiolitis	PCR (NP swabs)	14 (64%)	No control	2 (9%)	Greenberg *et al*. (2003)
Adults/children (USA) 188	Bronchiolitis, Pneumonia	Serology (IgG) ($n = 69$)	14 (18%)	Not significant	NA	Kumar *et al*. (2005)
	Asthma	PCR (NPl swabs) ($n = 169$)	29 (17%)	Not significant	NA	
Adults (UK) 29	RTI	Serology (IgG)	18 (62%)	Not significant	NA	Friedman *et al*. (2006)
		Serology (IgA)	5 (17%)	$P = 0.004$	NA	
Children (UK) 222	Bronchiolitis	Culture and/or PCR (NP swabs)	111 (50%)	No control	NA	Friedman *et al*. (2006)
Children (Italy) 101	CAP	Serology (IgM, IgG)	20–30%	No control	2 (2%)	Fasoli *et al*. (2008)
Children (Finland) 174	CAP	Serology (IgM)	18 (10%)	No control	6 (3.4%)	Heiskanen-Kosma *et al*. (2008)
Children (Brazil) 184	CAP	Serology (IgM, IgG)	3 (1.6%)	No control	1 (0.5%)	Nascimento-Carvalho *et al*. (2009)

*Recent infection was defined as: serological evidence for recent infection (positive IgM or significant increase between initial and convalescent IgG titres) or positive PCR result during the course of infection.
COPD, chronic obstructive pulmonary disease; NA, data not available.

high prevalence (23.5%) of antibody titers against *S. negevensis* among adults and children in this population, suggesting that these *Chlamydia*-related bacteria are simple colonizers (Kumar *et al*., 2005). Thus, thorough evaluation of the pathogenic role of *S. negevensis* is warranted, as most studies did not include a control group, and among the few studies with a control group, most failed to demonstrate a significant correlation with lower RTIs (Table 2).

The implication of *S. negevensis* in other respiratory diseases, such as chronic cough or asthma, has also been investigated and could not be demonstrated conclusively (Johnsen *et al*., 2005; Kumar *et al*., 2005; Korppi *et al*., 2006). In one study, *S. negevensis* was detected by a PCR method in bronchoalveolar lavage samples of lung transplant recipients with a surprisingly high prevalence (97.5%, when compared with only 14.1% in other solid-organ transplant recipients; $P < 0.0001$) (Husain *et al*., 2007). Many of these patients did not have documented pneumonia and the pathogenic role of *S. negevensis* in this context thus remains unclear. The authors of this study postulated a possible role in acute graft rejection, although the analysis was underpowered to reach statistical significance. Like other *Chlamydia*-related organisms, *S. negevensis* may infect free-living amoebae such as *Acanthamoeba* which are widespread in water, including hospital water supplies (La Scola *et al*., 2002; Thomas *et al*., 2006). Hospitalized patients exposed to aerosolized particles, such as those undergoing mechanical ventilation, may thus be colonized or infected by these intracellular bacteria, whose pathogenic role in this setting has been poorly investigated (La Scola *et al*., 2002). However, it is worth noting that *in vitro* studies of the pathogenesis of *S. negevensis* have demonstrated its ability to infect human macrophages and to induce a host cell inflammatory response, supporting its potential ability to cause human infections and the need for further clinical investigations (Kahane *et al*., 2008, 2007).

Parachlamydiaceae

This family has drawn increasing attention in the last decade because of the potential pathogenicity of its first recognized member, *P. acanthamoebae*, which was identified as the cause of an outbreak of fever of undetermined origin occurring in Vermont in 1989 (Birtles *et al*., 1997). An amoeba of the *Acanthamoeba* genus was isolated from the water of a humidifier and was subsequently shown to be infected with a gram-negative bacterium termed Hall's coccus. This organism was characterized by comparative sequence analyses (Birtles *et al*., 1997) and was found to be similar to a *Chlamydia*-like endoparasite of *Acanthamoeba* (strain Bn9) previously identified in the nasal mucosa of healthy subjects (Amann *et al*., 1997). Hall's coccus and strain Bn9 were subsequently assigned to the species *P. acanthamoebae* within the *Chlamydiales* order (Everett

et al., 1999). The ability of *P. acanthamoebae* to replicate within free-living amoebae of the genus *Acanthamoeba* has been well described and is shown in Fig. 5.

Further studies reported a significantly higher rate of seropositivity for *P. acanthamoebae* among patients with RTIs when compared with healthy controls (Marrie *et al.*, 2001; Greub *et al.*, 2003b; Greub, 2009). However, serological evidence for a recent infection in the absence of other potential documented pathogens could be assessed in only a few cases, suggesting that these bacteria may account for < 1% of CAP (Marrie *et al.*, 2001; Greub *et al.*, 2003a) and about 8% of ventilator-associated pneumonia (VAP) (Greub *et al.*, 2003b) (Table 3). More recently, the use of a specific real-time PCR allowed the identification of *P. acanthamoebae* or a related species as the only potential pathogen in as many as 13% of children with bronchiolitis, suggesting that the pathogenic role of *Parachlamydiaceae* may be underestimated in some clinical settings (Casson *et al.*, 2008c). Moreover, experimental models have demonstrated the ability of *P. acanthamoebae* to enter and replicate within human macrophages (Greub *et al.*, 2003c, 2005) and pneumocytes (Casson *et al.*, 2006) and to cause pneumonia in mice (Casson *et al.*, 2008a). Molecular analyses also allowed the identification of other members of the family *Parachlamydiaceae* in respiratory samples of some cases of pneumonia (Corsaro *et al.*, 2002; Casson *et al.*, 2008b; Haider *et al.*, 2008). *Protochlamydia naegleriophila* was detected as the unique potential pathogen in an immunocompromised patient with lung infiltrate (Casson *et al.*, 2008b) and one case of CAP possibly attributed to *Protochlamydia amoebophila* has been reported (Haider *et al.*, 2008) (Table 3). Further clinical studies are warranted to better define the pathogenicity and the epidemiology of *P. acanthamoebae* and other *Parachlamydiaceae* among the causal agents of RTIs.

Other *Chlamydia*-related organisms

Culture- and molecular-based studies on human, animal or environmental samples have led to the identification of many new *Chlamydia*-related organisms, attesting to the rich biodiversity within the order *Chlamydiales* (Greub, 2009). New strains have been identified and assigned to different families according to phylogenetic analyses, whereas others failed to grow in culture and could only be described by comparative sequence analyses. Some of them have been documented by PCR methods in human respiratory samples, but their pathogenic role remains unknown (Ossewaarde & Meijer, 1999; Corsaro *et al.*, 2001; Haider *et al.*, 2008). Using a molecular approach targeting the 16S rRNA gene (a conserved gene in the genome of all bacteria), Haider *et al.* (2008) recently found DNA of *Chlamydia*-related organisms in about 1% of adult patients with CAP,

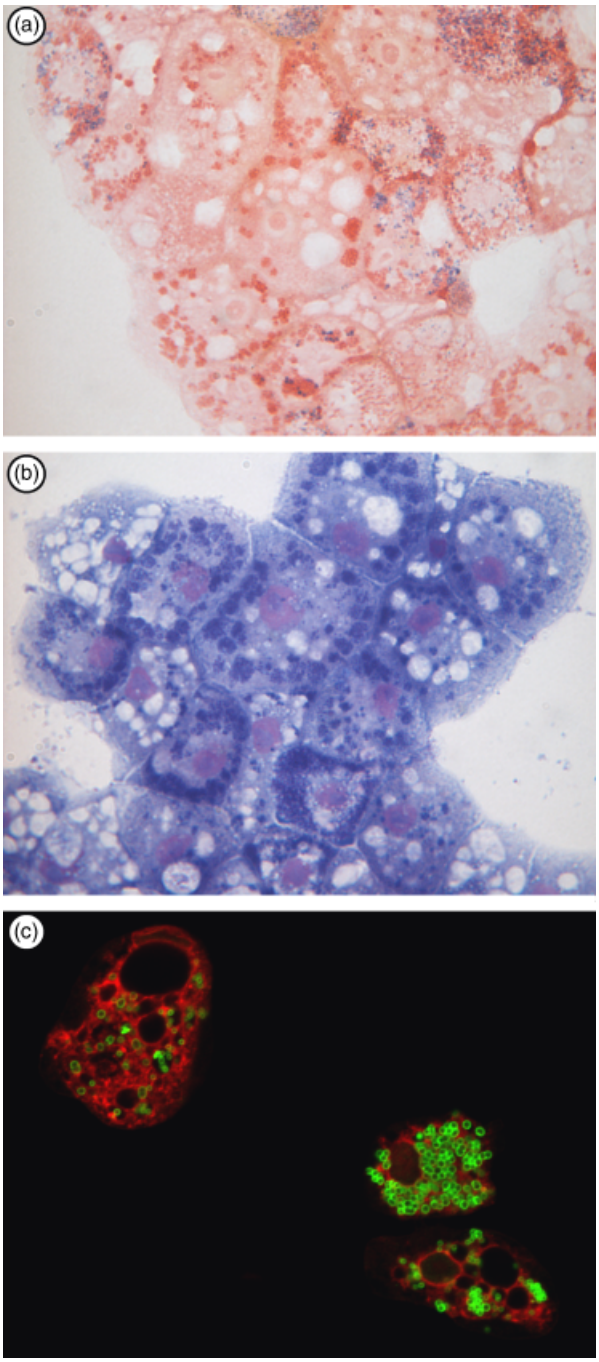

Fig. 5. *Parachlamydia acanthamoebae* within *Acanthamoeba castellanii*, as seen by gram staining (a), Diff-Quick staining (modified May–Grünwald Giemsa) (b), and immunofluorescence (c), respectively. Microscopy performed 24 h postinfection at an MOI of about × 10. (a) Note that elementary bodies are generally gram-positive, whereas the reticulate bodies are gram-negative. (c) The cell wall of *P. acanthamoebae* (in green) was stained with mice polyclonal anti-*Parachlamydia* antibodies whereas the amoeba was stained with concanavalin A. Magnification × 1000.

Table 3. *Parachlamydiaceae* as causal agents of pneumonia: review of the literature

Genus/species	Population (country) and number of patients	Disease	Diagnostic method	Positive results	*P* value (if controls)	Recent infection* and no alternative pathogen	References
Parachlamydia acanthamoebae	Adults (Canada) 371	CAP	Serology (IgM, IgG)	8 (2.2%)	$P < 0.01$	1 (0.3%)	Marrie *et al*. (2001)
	Adults/children (France) 1200	Pneumonia	PCR (BAL)	1 (0.1%)	No control	1 (0.1%)	Greub *et al*. (2003a)
	Adults (France) 37	VAP	Serology (IgM, IgG)	5 (13.5%)	$P < 0.001$	3 (8.1%)	Greub *et al*. (2003b)
	ICU adults (France) 210	Pneumonia	PCR and culture (BAL), serology (IgM, IgG)	3 (1.4%)	No control	1 (0.5%)	Berger *et al*. (2006)
	Children (Switzerland) 39	Bronchiolitis	PCR (NP swabs)	6 (15%)	No control	5 (13%)	Casson *et al*. (2008c)
Protochlamydia amoebophila	Adults (Austria) 387	CAP	PCR (respiratory samples)	1 (0.3%)	No control	1 (0.3%)	Haider *et al*. (2008)
Protochlamydia naegleriophila	Adults/children (Switzerland) 65	Pneumonia	PCR (BAL)	1 (1.5%)	Not significant	1 (1.5%)	Casson *et al*. (2008b)
Other *Parachlamydia* spp. (unclassified)	Adults/children (France/Italy) 170	Pneumonia	PCR (respiratory samples)	2 (1.2%)	No control	NA	Corsaro *et al*. (2002)

*Recent infection was defined as: serological evidence for recent infection (positive IgM or significant increase between initial and convalescent IgG titres) or positive PCR result during the course of infection.
BAL, bronchoalveolar lavage fluid; NA, data not available.

including *Waddlia chondrophila* (Family: *Waddliaceae*) and *Rhabdochlamydia porcellionis* (Family: *Rhabdochlamydiaceae*) (Haider *et al*., 2008). No sequence related to *S. negevensis* or *P. acanthamoebae* was detected in this series. The presence of *Rhabdochlamydia* spp. in respiratory samples of premature neonates has been recently reported, although their role as a causal agent of pneumonia or other systemic infections could not be assessed (Lamoth *et al*., 2009). It is thus difficult to estimate which proportion of RTIs in humans are caused by these *Chlamydia*-like organisms.

Waterborne mycobacteria

Nontuberculous mycobacteria are associated with disseminated or pulmonary infection in immunocompromised patients and may be the cause of various infectious diseases such as lymphadenitis, cutaneous infections or, rarely, pneumonia in the immunocompetent host (Falkinham, 1996). Among them, the *Mycobacterium avium* complex and *Mycobacterium kansasii* are the species most frequently associated with respiratory tract colonization or true infection in both immunocompromised and immunocompetent patients (Falkinham, 1996; Field & Cowie, 2006). *Mycobacterium xenopi*, *Mycobacterium fortuitum*, *Mycobacterium simae*, *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium gordonae* and *Mycobacterium malmoense* are other recognized agents of pneumonia, occurring mainly in immunocompromised hosts or patients with underlying pulmonary diseases (Table 4) (Field & Cowie, 2006).

The genus *Mycobacterium* has been considerably enriched with the discovery of a large number of new species during the last decades (Primm *et al*., 2004). These mycobacterial species have been isolated from water sources, soil, air, human or animal reservoirs, reflecting their ubiquity in the environment (Falkinham, 2002; Primm *et al*., 2004). Natural fresh or salt waters, such as lakes, rivers, swamps, estuaries or marine streams, are common habitats of opportunistic mycobacteria. Their resistance to chlorine and most disinfectants used for water treatment as well as their ability to survive despite low nutrient levels, low oxygen content or extreme temperatures allow them to colonize drinking water supplies, cooling towers, swimming pools and other recreational water systems (Falkinham, 2002; Black & Berk, 2003; Pagnier *et al*., 2009b). Their selection by surface disinfectants may also promote their widespread occurrence in the hospital environment. Moreover, these pathogens are able to form biofilms and to colonize medical devices such as bronchoscopes (Wallace *et al*., 1998; Falkinham, 2002). Nontuberculous waterborne mycobacteria are frequently isolated from clinical specimens (Martin-Casabona *et al*., 2004) and nosocomial outbreaks have been reported (Wallace *et al*., 1998; Phillips & von Reyn, 2001). In most cases, the presence of such microorganisms in the respiratory tract reflects transient colonization. However, several cases or small outbreaks of hospital-acquired pneumonia have been ascribed to *M. xenopi*, *M. chelonae* and *M. simae* (Wallace *et al*., 1998; Phillips & von Reyn, 2001; Conger *et al*., 2004). Community-acquired respiratory diseases such

Table 4. Mycobacteria other than tuberculosis (MOTT): review of the most frequent agents of pneumonia

Mycobacterium species	Pathogenic role in pneumonia	References
M. avium intracellulare complex	Most frequent cause of MOTT-associated respiratory infection (variable presentation)	Olivier (1998), Field & Cowie (2006), Kim *et al*. (2008), Parrish *et al*. (2008)
	More frequent in HIV and immunocompromised patients	Olivier (1998), Field & Cowie (2006), Parrish *et al*. (2008)
	Cause of hypersensitivity pneumonia after exposure to hot tubes	Embil *et al*. (1997), Field & Cowie (2006)
M. kansasii	Pulmonary disease similar to *M. tuberculosis*. One of the MOTT most frequently associated with pneumonia in both immunocompetent and immunocompromised hosts	Evans *et al*. (1996a, b), Campo & Campo (1997), Taillard *et al*. (2003), Maliwan & Zvetina (2005), Kim *et al*. (2008)
M. xenopi	One of the MOTT most frequently associated with pneumonia in both immunocompetent and immunocompromised hosts	Juffermans *et al*. (1998), Faress *et al*. (2003), Andrejak *et al*. (2007), Kim *et al*. (2008), van Ingen *et al*. (2008), Marusic *et al*. (2009)
M. malmoense	Most frequent cause of MOTT-associated respiratory infection in the United Kingdom and Sweden, especially in patients with underlying lung diseases. Cavitations are often present	Research Committee of the British Thoracic Society (2001), Henriques *et al*. (1994), Field & Cowie (2006)
M. chelonae	Rare cause of fever and pneumonia in neutropenic cancer patients	McWhinney *et al*. (1992), Levendoglu-Tugal *et al*. (1998), Peres *et al*. (2009)
	Rare cause of pneumonia in patients with oesophageal or swallowing disorders	Burke & Ullian (1977), Hadjiliadis *et al*. (1999)
M. fortuitum	Rare cause of pneumonia in patients with oesophageal or swallowing disorders	Howard *et al*. (1991), Hadjiliadis *et al*. (1999)
	Some cases of pneumonia reported mainly in immunocompromised patients	Marchevsky *et al*. (1982), Ellis & Qadri (1993), Al Shaalan *et al*. (1997), Abe *et al*. (1999), Miguez-Burbano *et al*. (2006)
M. simae	One of the MOTT most frequently isolated from respiratory samples	Valero *et al*. (1995), El Sahly *et al*. (2002), Samra *et al*. (2005)
	Cause of pneumonia in patients with underlying pulmonary diseases, rarely in AIDS patients	Bell *et al*. (1983), Huminer *et al*. (1993), Valero *et al*. (1995), Maoz *et al*. (2008)
M. gordonae	Frequently isolated from sputum, but rare cause of respiratory infection	Eckburg *et al*. (2000), Thomsen *et al*. (2002), Field & Cowie (2006)

as hypersensitivity pneumonitis and even cases of true pneumonia have been reported in healthy individuals exposed to aerosols from different water sources (spas, swimming pools, hot tubs, metalworking fluid) (Embil *et al*., 1997; Primm *et al*., 2004).

Mycobacteria and free-living amoebae thus share the same ecosystem and their close interaction has been suspected since the presence of mycobacteria within an amoebal host was first reported in 1973 (Jadin, 1973). Further *in vitro* studies not only demonstrated that mycobacteria were able to enter and replicate within the trophozoites and cysts of amoebae or other protozoa (Krishna Prasad & Gupta, 1978; Cirillo *et al*., 1997; Steinert *et al*., 1998; Strahl *et al*., 2001; Taylor *et al*., 2003; Adekambi *et al*., 2006; Mura *et al*., 2006; Whan *et al*., 2006; Thomas & McDonnell, 2007), but also that amoeba-grown mycobacteria displayed increased virulence in macrophage and mouse models of infection (Cirillo *et al*., 1997). Their recovery in hospital water networks has been strongly associated with the presence of amoebae (Thomas *et al*., 2006). Moreover, pathogenic strains of *M. kansasii* exhibit a better ability to grow in *Acanthamoeba castellanii* than the nonpathogenic strains colonizing the respiratory tract (Goy *et al*., 2007). The mechanisms involved in the pathogenicity of *M. avium* with respect to macrophages and amoebae have been found to be very similar (Danelishvili *et al*., 2007). These findings suggest that mycobacteria, like legionellae, may take advantage of amoebae, using them as a reservoir and as an evolutionary niche for the development of virulence factors. In contrast to legionellae and to some *Chlamydia*-like organisms residing within the cytosol of the amoebal cysts, mycobacteria are located within the double layers of the cysts (Steinert *et al*., 1998). This evolutionary adaptation may allow them to survive in a hostile environment such as phagocytic human cells and to resist to high chlorine concentrations or to the action of antibiotics (Miltner & Bermudez, 2000; Adekambi *et al*., 2006; Thomas & McDonnell, 2007).

Bradyrhizobiaceae

Afipia spp. and *Bosea* spp. are recently discovered gram-negative bacteria belonging to the class of *Alphaproteobacteria* (family: *Bradyrhizobiaceae*) and somehow related to

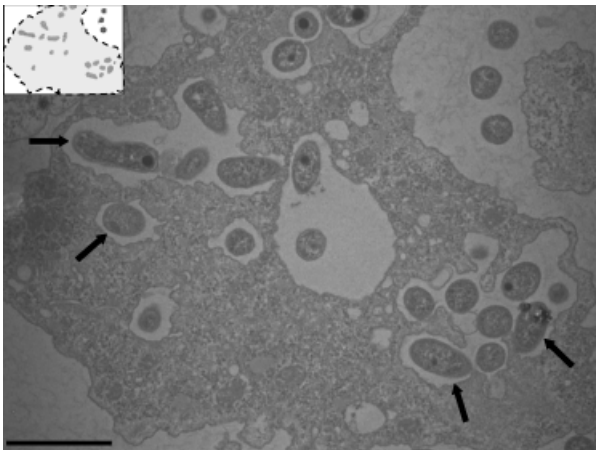

Fig. 6. *Bosea sequanensis* (arrows) within *Acanthamoeba castellannii*, as seen by electron microscopy. Magnification × 7000. Scale bar = 2 μm. The inset is a drawing showing the limits of the amoeba (discontinuous line) that contains about 19 bacteria (light grey); three bacteria that are localized outside of the amoeba are highlighted in dark grey.

Brucella spp. and *Bartonella* spp. (Brenner *et al*., 1991; Das *et al*., 1996). These *Bradyrhizobiaceae* are able to resist to amoebal microbicidal effectors (Fig. 6). The original isolation of *Afipia* spp. in clinical specimens such as lymph nodes (*Afipia felis*) or bone biopsy (*Afipia clevelandensis*) raised the question about their pathogenic role in human diseases, particularly in cat scratch disease (English *et al*., 1988; Brenner *et al*., 1991; Hall *et al*., 1991). However, subsequent analyses identified *Bartonella henselae* as the causal agent of this latter disease and the precise pathogenic role of *Afipia* remained undetermined (Jerris & Regnery, 1996). These bacteria were first detected in human respiratory samples by Brenner *et al*. (1991), who described the new genus *Afipia*. Drancourt *et al*. (1997) detected the presence of antibodies against *A. clevelandensis* in 1.5% of sera tested in the French national centre for rickettsial diseases and postulated a cross-reactivity between this bacterium and *Brucella* spp. or *Yersinia* spp., as about half of the cases had a diagnosis of certain or probable brucellosis or yersiniosis. However, about 15% of patients from this series were diagnosed as having pneumonia (Drancourt *et al*., 1997). The subsequent isolation of *Afipia* spp. and *Bosea* spp. in hospital water supplies suggests a possible role in nosocomial pneumonia (La Scola *et al*., 2000, 2002, 2003b, c; Thomas *et al*., 2006, 2007). Sero-epidemiological analyses revealed evidence of exposure to *Afipia* spp. and closely related *Alphaproteobacteria* in 13% of patients with hospital-acquired pneumonia, whereas no specific antibodies were detected in healthy blood donors ($P < 0.01$) (La Scola *et al*., 2002). The same strains were simultaneously detected in the water supplies of the intensive care units where these patients were staying. In another series of 30 patients in a single intensive care unit in France, seroconversion to *Afipia* spp. and *Bosea* spp. was documented in 17% and 20% patients with VAP, respectively (La Scola *et al*., 2003b). No alternative potential pathogen was documented in about half of these cases. This study also reported a case of pneumonia with detection of *Bosea massiliensis* by PCR in bronchoalveolar lavage fluid associated with seroconversion to the same microorganism (La Scola *et al*., 2003b). In another analysis of 210 ICU patients with pneumonia, *Bosea* spp. were detected in eight (3.8%) patients by culture, PCR or serological testing (*B. massiliensis* and *Bosea thiooxidans*) (Berger *et al*., 2006). However, evidence for recent infection was documented in only one case and potential alternative pathogens of pneumonia were isolated in all cases. All patients but one had hospital-acquired pneumonia. *Afipia* spp. were not detected in this population (Berger *et al*., 2006). A *Bosea* and some other *Alphaproteobacteria* have also been isolated from nasal swabs of hospitalized patients by amoebal coculture, although their pathogenic role was unclear (Greub *et al*., 2004b). Interestingly, the possible implication of the *Bradyrhizobiaceae* in CAP has not yet been investigated. *Bosea* strains have also been isolated from environmental sources of water other than hospital networks such as river water or drinking water plants (Rapala *et al*., 2006; Thomas *et al*., 2007) and *Afipia* spp. were the most common bacterial species found in biofilms from a dental unit water system in Baltimore (Singh *et al*., 2003). The actual role of *Bradyrhizobiaceae* in RTIs thus remains difficult to assess on the basis of serological diagnostic tools, which lack sensitivity and specificity, and in the absence of microbiological documentation in clinical specimens in most cases.

Biodiversity of amoebae-resisting microorganisms and perspectives for further investigations

Free-living amoebae represent a widespread evolutionary niche that may favour the selection of virulence traits in intra-amoebal bacteria, enabling them to survive in other phagocytic cells, including alveolar macrophages, which are one a major line of immune defence against invading pathogens. The examples of emerging pathogens provided in this review probably represent only the tip of the iceberg, and there is still a largely underestimated biodiversity of amoebae-resisting bacteria, which may have acquired their ability to cause diseases in humans by the development of virulence traits during their intra-amoebal life. This huge and so far unexplored biodiversity not only includes members of the clade presented in this review, i.e. *Legionella* spp., *Chlamydiae*, *Bradyrhizobiaceae* and mycobacteria, but also many other bacterial clades and giant viruses (Greub & Raoult, 2004). At least one of the amoebae-resisting viruses

Box 1. Mimivirus, a giant virus likely involved in lower respiratory tract infections

Mimivirus (*Mimiviridae*) is a double-stranded DNA virus belonging to the nucleocytoplasmic large DNA viruses (NCLDV). Initially discovered within free-living amoebae recovered by amoebal enrichment from a cooling tower during the investigation of an outbreak of community-acquired pneumonia in Bradford (UK), this microorganism was first considered to be a gram-positive bacterial coccus (La Scola *et al.*, 2003a). However, electron microscopy suggested that it was a giant virus of about 400 nm in diameter and subsequent studies unequivocally confirmed its affiliation within the NCLDV, which also includes the *Iridoviridae*, the *Phycodnaviridae*, the *Asfarviridae* and the *Poxviridae* (Koonin, 2005). Its genome of about 1.18 Mb encodes for about 911 ORFs and six tRNA genes (Raoult *et al.*, 2004).

As this virus was shown to grow well within *Acanthamoeba polyphaga* (La Scola *et al.*, 2003a), its possible resistance to destruction by human macrophages was readily suspected and confirmed by Ghigo *et al*. (2008). Thus, mimivirus was shown to selectively enter within human macrophages and not into epithelial cells (Ghigo *et al*., 2008). More importantly, entry occurred by phagocytosis (as demonstrated by its inhibition by overexpression of a dominant-negative form of a regulator of phagocytosis, dynamin-II), and was followed by efficient exponential replication (Ghigo *et al*., 2008). This further supported the paradigm that intra-amoebal pathogens may also be resistant to macrophages and suggested its possible pathogenic role towards humans.

Some clinical studies have further supported the possible role of mimivirus in lower respiratory tract infections. Thus, 36 (9.7%) patients with community-acquired pneumonia exhibited antibody reactivity against mimivirus as compared with 12 (2.3%) of 511 healthy controls ($P < 0.01$) (La Scola *et al.*, 2005). In addition, serologic evidence of mimivirus infection was also observed in five (19.2%) of 26 intensive care unit patients, whereas none of the 50 control patients were seropositive for mimivirus ($P < 0.01$). More importantly, mimivirus DNA was detected in a bronchoalveolar lavage sample from a 60-year-old comatose patient who presented two episodes of hospital-acquired pneumonia during hospitalization in the intensive care unit (La Scola *et al.*, 2005). Moreover, in another study, patients exhibiting antimimivirus antibodies had longer durations of mechanical ventilation and intensive-care unit stay, with median excesses of 7 and 10 days, respectively (Vincent *et al.*, 2009). However, when testing 496 different pneumonia patients with two different real-time PCRs, other investigators failed to identify any case of mimivirus (Dare *et al*., 2008). This discrepancy might potentially be due to the possible occurrence of mimivirus-like strains, which cross-react with mimivirus but exhibit some differences in the region of the viral helicase and thiol-oxidoreductase genes used as PCR targets, preventing their detection using this method (Dare *et al*., 2008). It is noteworthy that the pathogenic role of mimivirus has been clearly demonstrated by an accidental exposure of a laboratory technician to mimivirus (Raoult *et al*., 2006). Besides strongly supporting the pathogenicity of mimivirus, this laboratory-acquired infection highlights the importance of cautious manipulation of such emerging potential pathogens, especially given the current absence of antivirals that are efficient against such giant viruses.

Finally, the pathogenic role of mimivirus was further supported by a mouse model of infection (Khan *et al*., 2007). Thus, mice inoculated through the intracardiac route presented pneumonia similar to viral pneumonia due to measles, smallpox and rubella. Interestingly, a virophage called Sputnik was found closely associated with a new giant virus also belonging to the *Mimiviridae* (La Scola *et al*., 2008). Both the giant virus and the virophage may replicate efficiently within *Acanthamoeba* amoebae (Fig. 7). Whether this small icosahedral virus may modify the pathogenicity of the *Mimiviridae* remains to be investigated.

In conclusion, humans are commonly exposed to mimivirus or cross-reacting agents and mimivirus was pathogenic at least towards humans following accidental laboratory exposure and to mice following intravenous challenge. Moreover, this amoebae-resisting virus (or a related cross-reactive species) should be considered as an emerging agent of both nosocomial and community-acquired pneumonia and clearly merits further study.

discovered so far, the mimivirus, is also resistant to human macrophages and may be involved in lower RTIs, as suggested by clinical studies and by a well documented laboratory-acquired infection (see Box 1).

The research community should thus be aware of the wide biodiversity of amoebae-resisting microorganisms including novel *Chlamydiae*, new LLAP, some *Bradyrhizobiaceae*, the recently described novel waterborne mycobacterial species and giant viruses. Although *Parachlamydia* and some other amoebae-resisting *Chlamydiae* have already been investigated for their pathogenic potential (Corsaro & Greub, 2006; Greub, 2009), there is still an infinite and exciting perspective for further investigations with regard to the development of new diagnostic tools and the comprehension of the pathogenic roles and the cell biology of such microorganisms.

Further investigations may be especially important given the fact that the intra-amoebal environment may select strains that are preferentially resistant to antibiotics and to biocides. Such resistance may be due to the partial protection conferred by amoebal trophozoites and cysts, or may result from the acquisition of efflux mechanisms following exposition to heavy metals and other toxic compounds when inside the amoebal host. The concomitant resistance to biocides and chemical compounds that was first described in mycobacteria (Miltner & Bermudez, 2000), as well as the unexpected resistance of *Chlamydiae* to quinolones (Maurin *et al*., 2002; Casson & Greub, 2006; Goy & Greub, 2009), are illustrations of the potential of amoebae-resisting bacteria to select new virulence traits. Moreover, the intra-amoebal environment likely represents an important niche for gene exchange between intracellular pathogens, as exemplified by the occurrence in *Rickettsia bellii* (Ogata *et al*., 2006) and in *P. amoebophila* (Greub *et al*., 2004a) of similar genes encoding a putative F-like conjugative DNA transfer system. Such gene exchanges not only occur between different bacterial clades, but also likely take place with giant amoebae-resisting viruses and with the genomic content of the amoebal host itself, explaining the relatively large genomes of amoebae-resisting microorganisms.

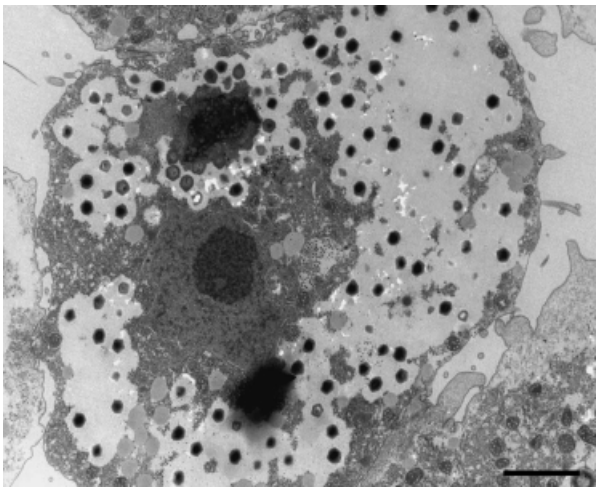

Fig. 7. Presence of numerous viral particles (mimivirus) within the *Acanthamoeba polyphaga* amoeba. Note the presence in the middle of the picture of very small viral particles corresponding to the Sputnik virus. Scale bar = 2 μm.

Conclusion

Amoebae and intra-amoebal microorganisms have coevolved for millions of years and have generated a wide biodiversity of microorganisms that are likely to be able to resist both the phagocytic machinery of amoebae and human macrophages. Thus, the amoebal evolutionary crib may have produced a widespread biodiversity of potential pathogenic species that remain to be discovered. The isolation of these fastidious bacteria and their species identification by culture-based methods, such as amoebal coculture and the amoebal enrichment, as well as the development of molecular methods for their detection in clinical samples, are warranted for a better assessment of their actual role in human diseases such as pneumonia, which remains a major cause of morbidity and mortality in the world. A better comprehensive approach of the interactions between free-living amoebae and amoebae-resisting organisms may give further insights into the mechanisms of pathogenicity of microorganisms and their mode of acquisition of resistance to environmental aggressions, such as phagocytosis by amoebae and macrophages, biocides or chemical compounds, with potential implications for therapeutic approaches.

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