Parachlamydiaceae: Potential Emerging Pathogens

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Parachlamydiaceae, which naturally infect amoebae, form a sister taxon to the Chlamydiaceae on the basis of the Chlamydia-like cycle of replication and 80% to 90% homology of ribosomal RNA genes. Because intra-amoebal growth could increase the virulence of some intracellular bacteria, Parachlamydiaceae may be pathogenic. Arguments supporting a pathogenic role are that Chlamydia pneumoniae, a well-recognized agent of pneumonia, was shown to infect free-living amoebae and that another member of the Chlamydiales, Simkania negevensis, which has 88% homology with Parachlamydia acanthamoebae, has caused pneumonia in adults and acute bronchiolitis in infants. The recent identification of a 16S rRNA gene sequence of a Parachlamydiaceae from bronchoalveolar lavage is additional evidence supporting potential for pathogenicity.

Nosocomial pneumonia, a frequent complication associated with considerable illness and death (1,2), is the leading cause of death from nosocomial infections (3). Community-acquired pneumonia, which is also common, is associated with a case-fatality rate of up to 8.8% (4). Despite use of standard diagnostic methods, no microbial cause could be identified in 47% to 55% of community-acquired pneumonia worldwide in adults (5–7) and 20% to 75% of nosocomial pneumonia (8,9). Emerging intracellular bacteria, which grow poorly or not at all on media used routinely for detecting human pathogens from clinical samples, could be the causative agents of these pneumonias of unknown etiology. During recent decades, several previously unrecognized intracellular bacteria have been discovered through the genotypic approach. In addition, use of amoebal coculture procedures (10) allows recovery of some fastidious gram-negative bacteria, such as the Legionella-like amoebal pathogens (11,12), Candidatus Odysella thessalonicensis (13), Sacrobium lyticum (14), several Afipia species (15), and Chlamydia-like endosymbionts (16,17).

Amoebae: Microbial Trojan Horses

Although Legionella was the first pathogen demonstrated to multiply and persist in amoebae (18), several other fastidious intracellular bacterial pathogens, including Chlamydia pneumoniae (19), Mycobacterium avium (20), Listeria monocytogenes (21), and an Ehrlichia-like organism (22), may infect free-living amoebae. Extensive study of the ecology of Legionella pneumophila has confirmed empirical observations of its predilection for growth in hot water tanks and its localization in sediment (23). Rowbotham described the ability of L. pneumophila to multiply intracellularly within protozoa (18) and suggested that free-living amoebae could be a reservoir for Legionella species (24). As amoebae are common inhabitants of natural aquatic environments and water systems (25,26) and are resistant to extreme temperatures, pH, and osmolarity conditions while encysted (27), the Legionella reservoir is important. Growth of free-living amoebae at high temperatures (44°C to 53°C) was observed more frequently for strains isolated from hot-water tanks (mainly Hartmanella vermiformis) than for those isolated from moist sanitary areas (mainly Acanthamoeba, Naegleria, and Valkhampfia species) (26). This great tolerance of cysts and species-dependent thermotolerance of trophozoites could account for the difficulty in eliminating Legionellae from water systems (28). The resistance of Acanthamoeba spp. cysts to various disinfecting solutions (29–31) complicates the eradication of free-living amoebae. Moreover, a wide variety of Enterobacteriaceae have increased resistance to chlorination when ingested by Tetrahymena pyriformis (32). Thus, free-living amoebae could readily act as Trojan horses for bacterial endosymbionts (33,34).

The relationship between Legionellae and free-living amoebae, which serves as a model for other endosymbionts such as Parachlamydiaceae, is not restricted to the role of reservoir. Indeed, Acanthamoeba strains were found to produce Legionella-containing vesicles, which may be agents of transmission of legionellosis. The risk of transmission may be underestimated by plate count methods (35). In addition, Legionellae grown inside amoebae were more virulent (36,37), more motile (24), and more resistant to biocides (38) than are bacteria cultured in axenic media. The entry of Legionellae into monocytes was found to be enhanced by the intra-amoebal growth environment (39). In addition, intra-amoebal growth of L. pneumophila was shown to induce an antibiotic-resistant phenotype, while Legionellae cultured in broth did not (40). Similarly, M. avium living within Acanthamoeba had greater resistance to rifabutin, clarithromycin, and azithromycin than did strains living in macrophages (41). This finding could result from decreased uptake of antibiotics into the amoebae, an inactivation of the compound within amoebae, or a change in the bacterial phenotype. Replication of bacteria in amoebae was found not only to affect the bacterial host (through increased potential for spread, resistance to biocides and antibiotics, and acquisition of virulence traits) but also to enhance the pathogenicity of the free-living amoebae (42).
The Parachlamydiaceae

These Chlamydia-like endosymbionts are small Gimenez-stained (43) coccoid bacteria (Figure 1) that naturally infect amoebae and are inconsistently stained with Gram stain. Electron micrographs of Acanthamoeba demonstrate the presence of bacteria at different developmental stages typical of the Chlamydiales, such as elementary and reticulate bodies (Figure 2). A new Parachlamydiaceae family was proposed (44) that forms a sister taxon to the Chlamydiaceae, as it has a Chlamydia-like cycle of replication and 80% to 90% homology of ribosomal RNA genes. This family comprises two genera, of which the type strains are Parachlamydia acanthamoebae (17) and Neochlamydia hartmanellae (45). Members of the Parachlamydiaceae were proposed to have at least 95% homology of the 16S or 23S rRNA genes with P. acanthamoebae (44). However, comparison of the 16S rRNA gene sequences of four additional Parachlamydia with P. acanthamoebae showed substantial phylogenetic diversity within this genus (Figure 3), with 91.2% to 93.1% 16S rRNA gene sequence homology with P. acanthamoebae (46). The ecological loci and prevalence of the Parachlamydiaceae are unknown, but the latter could be underestimated, as this fastidious gram-negative bacteria was recovered only by amoebal cultures, a procedure not performed routinely on clinical samples. Moreover, these Chlamydia-like organisms have potential for widespread dissemination, as they are mostly endosymbionts of Acanthamoeba, a free-living amoeba with worldwide distribution (27).

Strains of Parachlamydiaceae

Nine strains of Parachlamydia have been described (Table). The first, P. acanthamoebae, was identified within Acanthamoeba BN9, an amoeba recovered from the nasal mucosa of a female volunteer (17). Its 16S rRNA sequence had 88.2% homology with Simkania negevensis and 87% homology with Chlamydophila pneumoniae (17). The second, Berg17 endosymbiont, also isolated from the nasal mucosa of a female volunteer, seems to have an rRNA signature similar to that of the BN9 endosymbiont, as demonstrated by the binding of the BN9 hybridization probe designed for in situ identification of P. acanthamoebae (17). The third, Hall’s coccus, was found in an Acanthamoeba isolated from water taken from a humidifier in a case of humidifier-associated fever in Vermont (16). Its 16S rRNA gene sequence had >99% similarity with that of BN9 endosymbiont and 86% to 87% with those of four Chlamydia species (16). Two additional Parachlamydiaceae, UWE1 and UWE25, were also found to infect Acanthamoeba. Both amoeba strains were recovered from soil samples from Washington State (46). A sixth strain, UWC22 endosymbiont, infected an Acanthamoeba recovered from infected corneal tissues (46). TUME1 endosymbiont was found in an amoeba recovered from municipal sewage sludge in Germany (46). The eighth strain, Neochlamydia hartmanellae, is the only strain of Parachlamydiaceae isolated from Hartmanella vermiformis. It did not grow on Acanthamoeba sp. or Naegleria, and its 16S rRNA gene sequence had only 92% homology with that of P. acanthamoebae and varied from 91.6% to 97.1% with the four latter endosymbionts of Acanthamoeba (45). The last one, CorvenA4, could not be isolated. Only its 16S rRNA sequence was retrieved from a respiratory sample (47).
SYNOPSIS

Pathogenicity

Rationale for Potential Pathogenicity

Intra-amoebal growth may increase the virulence of some intracellular bacteria (39), prompting concern that other intracellular bacteria recovered from amoebae, such as the *Parachlamydiaceae*, could be pathogenic. Indeed, a bacterium able to survive exposure to the lytic enzymes of amoebal phagolysosomes would probably also survive the lytic activity of macrophages. This hypothesis is supported by the fact that mutants of *Legionella* that have similar cytotoxic defects and intracellular replication in mammalian macrophages and protozoa have been isolated (48), suggesting a common adaptive mechanism to the intracellular environment. Moreover, *Parachlamydia* can adapt to mammalian cells, as demonstrated by successful passage from an amoebal host to Vero cells (a monkey cell line) (17). Additional arguments in favor of a pathogenic role of the *Parachlamydiaceae* are that *Chlamydia pneumoniae*, a well-recognized agent of pneumonia, was shown to infect free-living amoebae (19) and that another member of the *Chlamydiaceae*, *Simkania negevensis* (49,50), which has 88% homology with *P. acanthamoebae* (46), has been shown to cause pneumonia in adults and acute bronchiolitis in infants (51,52).

Strong evidence that some *Parachlamydiaceae* could be pathogenic came from the identification of Hall’s coccus in an amoeba isolated from the source of an outbreak of humidifier-associated fever in the United States, as well as related serologic studies (16). In a study of 500 patients with pneumonia, fourfold rising titers against Hall’s coccus were observed in two patients and convalescent-phase antibodies in three others (53). In a second study, two patients had convalescent-phase antibodies (16). These results were recently confirmed: 8 (2.2%) and 3 (0.8%) of 371 patients with community-acquired pneumonia were seropositive (titer >1/50) or had a fourfold rise in *Parachlamydia* antibody titers compared with none of 511 healthy study participants (54). The recent identification of a 16S rRNA gene sequence of *Parachlamydiaceae* from bronchoalveolar lavage provides additional evidence of potential pathogenicity (47). However, the contamination of this specimen by an amoeba harboring the *CorvenA4-Parachlamydiaceae* could not totally be ruled out. These findings should be interpreted cautiously as water contamination probably led to the initial false attribution of *Afipia felis* as the causative organism of cat-scratch disease (55). The identification in respiratory tract specimens of three new *Chlamydia*-like strains, which had phylogeny closer to that of the *Parachlamydiaceae* and *Simkaniaceae* than the *Chlamydia* and *Chlamydophila* (56), is an additional argument in favor of a role of the *Parachlamydiaceae* in the pathogenesis of respiratory diseases.

In addition, a patient with adult Kawasaki syndrome was found to have a fourfold rise in antibody titer to *P. acanthamoebae* (54). A possible relationship between a previous respiratory infection and Kawasaki syndrome has already been reported (57,58). Thus, the role of *Parachlamydia* in the pathogenesis of Kawasaki syndrome should be explored further.

As *Parachlamydia* could potentially be resistant to lytic macrophages enzymes for years, it could enhance chronic inflammatory disease or chronic pathogenic mechanisms, such as the one leading to vascular damage. A role of *Parachlamydiaceae* in the pathogenesis of arteriosclerosis is suggested by

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**Figure 3.** Neighbor-joining phylogenetic tree of the 16s rRNA gene sequence of *Chlamydiaceae*, including *Chlamydiaceae*, *Parachlamydiaceae*, and *Simkaniaceae*, compared with *Legionella pneumophila* (M59157) as outgroup. Bar represents estimated evolutionary distance. The numbers at each node are the results of bootstrap analysis; each value is derived from 100 samples.
the presence in an abdominal aneurysm specimen of a Chlamydia-like strain that had a sequence closer to that of P. acanthamoeba than to Chlamydia, Chlamydophila, and Simkaniaeae (56). Some serologic studies have suggested that Chlamydophila pneumoniae could play a role in the pathogenesis of arteriosclerosis (59,60), although this observation was not confirmed in other studies (61,62). Such a discrepancy might result from serologic cross-reactions or confounding by a pathogen such as Parachlamydia, which in light of its homology could share epitopes, mode of transmission, or both with C. pneumoniae.

Based on this rationale, one may hypothesize that some Parachlamydiaceae could cause pneumonia. Thus, patients with nosocomial or community-acquired pneumonia of unknown etiology should ideally receive an extensive diagnostic work-up, including testing for Parachlamydia. In addition, patients with arteriosclerosis and Kawasaki disease or other infectious syndromes of unknown etiology should perhaps be tested for Parachlamydia. As Parachlamydia strains were all identified within free-living amoebae, recent history of swimming in ponds, rivers, or swimming pools might prompt a specific diagnostic approach.

### Diagnostic Methods

No diagnostic tool is commercially available. Because of the fastidious nature of Parachlamydiaceae, molecular biology is probably the easiest and cheapest diagnostic approach. Serologic testing is also promising; however, it requires antigen and a laboratory capable of performing amoebal coculture. Serologic results may be useful for epidemiologic studies, as they may provide information on past or present contact with the antigen. Both molecular and serologic methods may yield results in <24 hours.

Although time-consuming, culture-based diagnostic methods have the advantage of enabling the recovery of strains. These methods encompass two main approaches. The first one directly targets the recovery of Parachlamydiaceae, with amoebae used as cell background. A convenient broth for amoebal coculture is Page’s modified Neff’s amoeba saline (PAS) (10), which is preferable to Nelson’s and peptone-yeast-glucose medium because PAS is devoid of nutrients, thus reducing overgrowth of potential contaminants in clinical samples. Although incubation at 37°C may be ideal for bacterial recovery, lower temperatures (30°C–35°C) are generally used to prevent amoebal death or encystment (12,13,20). The coculture should be examined regularly for amoebal lysis or encystment (12,13,20). The coculture should be examined regularly for amoebal lysis or encystment (12,13,20). The coculture should be examined regularly for amoebal lysis or encystment (12,13,20).

### Future Directions

The role of Parachlamydia sp. as an emerging pathogen needs to be confirmed. In view of the genetic diversity of the Parachlamydiaceae (46), their phylogeny needs to be elucidated, as the various species could be associated with species-specific pathogenicity. Search for additional Parachlamydia strains in hospital water systems could help define potential nosocomial exposures. Because the Parachlamydiaceae are difficult to culture, simpler approaches are being developed, including serologic and molecular tests. These methods could

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### Table. Strains of Parachlamydiaceae

| Strain         | Sample, context and location | Host*                        | % 16S rRNA homology to BN9 | % 16S rRNA homology to C. pneumoniae | Ref |
|----------------|------------------------------|------------------------------|-----------------------------|--------------------------------------|-----|
| BN9 endosymbiont | Nasal swab of female volunteer, Germany | Acanthamoeba sp. strain BN9 | 100                         | 87.6                                 | 17  |
| Berg17 endosymbiont | Nasal swab of female volunteer, Germany | Acanthamoeba mauritanicensis | na                         | na                                   | 17  |
| Hall’s coccus  | Water sample, humidifier fever, Vermont | Acanthamoeba sp. | 99.6                       | 87.4                                 | 16  |
| UWE1 endosymbiont | Soil samples, Washington State | Acanthamoeba sp. strain UWE1 | 93.7                       | 86.6                                 | 46  |
| UWE25 endosymbiont | Soil samples, Washington State | Acanthamoeba sp. strain UWE25 | 93.2                       | 86.8                                 | 46  |
| UWC22 endosymbiont | Infected corneal tissues, Washington State | Acanthamoeba sp. strain UWC22 | 91.3                       | 87.3                                 | 46  |
| TUME1 endosymbiont | Municipal sewage sludge, Germany | Acanthamoeba sp. strain TUME1 | 91.0                       | 87.2                                 | 46  |
| Neochlamydia hartmannellae | Water system of a dental unit, Germany | Hartmanella vermiciformis | 91.5                       | 86.8                                 | 45  |
| CorvenA4       | Bronchoalveolar washing, France | na | 91.4                       | 85.0                                 | 47  |

*Bacterial strains were identified in free-living amoebae, isolated by culture on nonnutrient agar.

*Estimated with Clustal W (53) available on the website of Pole Bio-informatique Lyonnais, Lyon, France (http://pbil.ibcp.fr/).

*16S rRNA of Chlamydia pneumoniae strain N16 (GenBank accession number U68426).

*Berg17 endosymbiont was shown to have a similar rRNA signature from Bn9 endosymbiont (binding of the Bn9658 hybridization probe designed for in situ identification of Parachlamydia acanthamoebae); however, the 16S rRNA sequence of that strain is not available.

*Direct polymerase chain reaction amplification and sequencing from DNA extracted from the respiratory sample; no strain was isolated.
be performed on a large number of samples from both healthy and ill persons. Patients with community-acquired pneumonia, nosocomial pneumonia, Kawasaki disease, and arteriosclerosis should be tested. Increased resistance to antimicrobial drugs, which may be associated with intra-amoebal growth, is another promising area for future study.

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