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Pathogenic Potential of Novel Chlamydiae and Diagnostic Approaches to Infections Due to These Obligate Intracellular Bacteria

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INTRODUCTION

Chlamydiae are obligate intracellular parasites of vertebrates, of some arthropod species, and of several free-living amoebae (reviewed in references 14, 32, and 68). They exhibit a peculiar two-stage developmental cycle that includes an extracellular infectious elementary body and an intracellular vegetative reticulate body. A further infective stage, the crescent body, was recently described for the *Parachlamydiaceae* (41), a new family within the order *Chlamydiales*.

The family *Chlamydiaceae* comprises two genera, *Chlamydia* and *Chlamydophila* (29). While *Chlamydia* species seem to infect only mammals such as humans, rodents, and swine, host specificity for *Chlamydophila* species is less strict, including for amphibians, reptiles, birds, and mammals (14). Both *Chlamydia* and *Chlamydophila* species comprise important human pathogens (88). *Chlamydia trachomatis* is a common cause of urogenital infection in humans and the agent of trachoma, one of the leading infectious causes of blindness worldwide. *Chlamydophila pneumoniae* is another important human pathogen, causing mainly respiratory infections. *C. pneumoniae* might also be involved in the pathogenesis of atherosclerotic cardiovascular diseases (8) and neurodegenerative syndromes (91). The other species are mainly veterinary pathogens, though some of them may cause rare but severe anthropozoonotic infections, such as psittacosis

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due to *Chlamydophila psittaci* or zoonotic abortion due to *Chlamydophila abortus* (14, 68, 83).

Historically, the term "chlamydia-like organisms" has been applied to refer to any intracellular microorganism that, like *Chlamydiaceae*, exhibited a two-stage developmental cycle. Since phylogenetic molecular analyses performed on some of these chlamydia-like organisms have showed this group to be polyphyletic, the term "chlamydia-like organisms" is only descriptive and retains no taxonomic value. Other authors (55, 56) proposed the term "environmental chlamydiae" to refer to chlamydial organisms that fall outside the family *Chlamydiaceae*, with the latter being designated "pathogenic chlamydiae." However, such a distinction seems inadequate, since a growing body of evidence supports the pathogenic role of some of these chlamydiae (32, 40). By analogy to the term "amoeba-resistant microorganisms" (43), the term "amoeba-resisting chlamydiae" could also be applied to the species that may infect and survive within amoebae, i.e., *Parachlamydiaceae*, *Simkania negevensis*, and *Waddlia chondrophila* (see below). Indeed, most of these new species exhibited symbiotic or lytic interaction with amoebae. Nevertheless, since not all new species of chlamydiae have been tested for their ability to resist destruction by free-living amoebae, the more general term "novel chlamydiae" should be preferred to designate all the chlamydiae not belonging to the *Chlamydiaceae*.

These chlamydiae, which have recently been discovered and assigned to new families, are currently being investigated for their role as emerging pathogens. *Simkania negevensis* (family *Simkaniaceae*) (32) and *Parachlamydia acanthamoebae* (family *Parachlamydiaceae*) (40) could represent important respiratory pathogens in humans, while *Waddlia chondrophila* (family *Waddliaceae*) seems to be a new agent of abortion in ruminants (21, 53).

Molecular studies performed on human and animal samples have demonstrated the wide biodiversity and broad host range of chlamydiae. Thus, additional *Parachlamydiaceae* species, such as *Neochlamydia* sp., and a large variety of new 16S rRNA gene phylotypes have been detected in humans (15, 17), cats (93), Australian marsupials (7, 20), reptiles (6, 90), and fishes (24), as well as in various environmental samples (16, 18).

Moreover, new members of the *Chlamydiales* infecting invertebrates have recently been characterized. These include *Fritschea bemisiae* and *Fritschea eriococci* (family *Simkaniaceae*), which infect homopteran insects (28, 92), and *Rhabdochlamydia porcellionis* (64) and *Rhabdochlamydia crassificans* (19), which infect the woodlouse *Porcellio scaber* (Crustacea: Isopoda) and the cockroach *Blatta orientalis* (Insecta: Blattodea), respectively. The presence of chlamydiae in arthropods is interesting, since arthropods were not previously considered to play a role in the epidemiology of chlamydial infections, with the only exception being flies as a vector of the agent of trachoma (25, 26). To date, if we exclude cases of possible *Rhabdochlamydia*-related uveitis in humans (see below), there is no hint that these invertebrate-associated chlamydiae may be pathogenic to humans.

Evidence for a much larger biodiversity within *Chlamydiales* was afforded by several molecular studies performed on humans, animals, and environmental samples (Table 1). Here we report the present knowledge on the pathogenicity of all *Chlamydiales* except *Chlamydiaceae*, and we review the diagnostic methods currently available to diagnose human infections potentially due to these emerging pathogens.

PATHOGENIC POTENTIAL OF NOVEL CHLAMYDIAE

Human Infections

***Parachlamydiaceae*.** Humans are commonly exposed to *Parachlamydiaceae*, as demonstrated (i) by the presence of two strains of *Parachlamydia acanthamoebae* within amoebae isolated from the nasal mucosa of two healthy volunteers (1, 74, 77) and (ii) by the amplification of *Parachlamydia* sp. DNAs from nose and/or throat swabs (17, 73, 80).

There is much evidence supporting the role of *P. acanthamoebae* as an emerging respiratory pathogen (reviewed in reference 40). First, *Parachlamydia* strain Hall's coccus was discovered in an amoeba isolated from the source of an outbreak of fever (65), and a serological study identified an association with an acute infection (5). In another serological study, a fourfold increase in antibody titers against *P. acanthamoebae* was observed in 2 of 500 patients with pneumonia (4). In addition, 8 of 371 (2.2%) patients with community-acquired pneumonia exhibited antibodies directed against *P. acanthamoebae*, compared to 0 out of 511 healthy subjects ($P = 0.001$) (71). Two patients described in that study presented pneumonia and serological evidence of acute *P. acanthamoebae* infection (71). The first patient suffered from an adult-onset Kawasaki disease (acute vasculitis following respiratory infection). The second patient was a renal transplant recipient chronically treated with cyclosporine and corticosteroids. This seroconversion in an allograft recipient treated with immunosuppressive drugs (71) and the fact that another case of probable *Parachlamydia* pneumonia was identified in a human immunodeficiency virus-infected subject with only 80 CD4 cells/mm^3 (51) suggest that *P. acanthamoebae* is an opportunistic human respiratory pathogen. Recently, significant antibody titers against *P. acanthamoebae* have been detected in 5 of 37 (13.5%) polytraumatized intensive care patients, while serology was negative in 100 blood donors ($P = 0.001$) (48). In that work, which highlights the high prevalence of *P. acanthamoebae* infection in intensive care unit patients, seroconversion was associated with aspiration pneumonia. The temperature-dependent release of *P. acanthamoebae* (45) further supports the role of this amoeba-resisting chlamydia in this clinical setting. The amplifications of *Parachlamydia* sp. DNA from bronchoalveolar lavage fluid and sputum are additional hints of a potential pathogenicity (15, 17, 51). Finally, *P. acanthamoebae* may enter and multiply within human macrophages (44, 49, 50). These studies suggest that human exposure to *Parachlamydiaceae* spp. may lead to bronchitis, community-acquired pneumonia, and aspiration pneumonia. No animal model of infection has yet been established, and further studies are needed to better define the role played by *Parachlamydiaceae* as agents of pneumonia.

The pathogenic role of *Neochlamydia hartmannellae* (57) is unknown and remains to be determined. However, this microorganism could play a role in ocular infections, as *Neochlamydia* sp. strain UWC22 has been recovered within an *Acanthamoeba* isolated from a contact lens of a patient with keratitis (36, 37). Whether this role is direct, with a pathogenesis similar to that of infections due to other ocular chlamydiae such as *C. tracho-*

TABLE 1. Detection of novel *Chlamydiales* from human and animal samples

Host(s)[a]	Sample(s)[b]	Clinical syndrome(s)	Detection[c]	Chlamydia(e)[d]	Reference(s)
Human	Respiratory	Respiratory infections	PCR, serology	*P. acanthamoebae*	5, 40, 45, 48, 51, 71
			PCR	*Parachlamydiaceae*	15, 17
			Isolation, PCR, serology	*S. negevensis*	31, 33, 34, 39, 60, 66, 67, 81
			PCR	Novel lineages	73
	Ocular	Conjunctivitis/keratitis, uveitis	Isolation, PCR	*Parachlamydiaceae*	37, 73
		Uveitis	PCR	*Rhabdochlamydia* sp.	14, 73
		—[e]	PCR	Novel lineages	73
	Blood, artery	—	PCR	*Parachlamydiaceae*	73
		—	PCR	*Simkania* sp.	73
		—	PCR	Novel lineages	73
Mammals (Placentals)					
Cat	Conjunctival swab	Conjunctivitis/keratitis	PCR	*Neochlamydia* sp.	93
Swine	Artery	—	PCR	*Parachlamydiaceae*	73
Bovine	Fetus	Abortion	Isolation, PCR, serology	*Waddlia chondrophila*	21, 22, 53
Fruit bat	Urine	—	Isolation, PCR	*Waddlia malaysiensis*	9, 10
Australian marsupials	Conjunctival swab	Conjunctivitis	PCR	*P. acanthamoebae*	7
			PCR	*Parachlamydiaceae*	7
			PCR	Novel lineages	7
	Urogenital, kidney, bladder	Cystitis, nephritis	PCR	*Parachlamydiaceae*	7, 20
			PCR	*Waddlia* sp.	7
	Ocular	Conjunctivitis	PCR	Novel lineages	7, 20
	Respiratory	—			
Birds (chicken)	Urogenital	—	PCR	Novel lineages	73
Reptiles					
Green sea turtle	Heart tissue	—	PCR	*Neochlamydia* sp.	6
Various species	Various tissues	Granulomatous inflammation	PCR	*Parachlamydia*/*Simkania*	90
Fishes (various species)	Gills	Epitheliocystis	PCR	*Neochlamydia* sp.	73
			PCR	Novel lineages	73
			PCR, LPS	*Piscichlamydia salmonis*	24
			LPS	Uncharacterized chlamydia-like	52
Insects					
Bemisia tabaci (Homoptera)	Bacteriocyte	Lower fecundity and plant pathogenicity[f]	PCR	*Fritschea bemisiae*	28, 92
Eriococcus spurius (Homoptera)	Bacteriocyte		PCR	*Fritschea eriococci*	28, 92
Blatta orientalis (Blattoidea)	Fat body	Abdominal swelling	PCR	*Rhabdochlamydia crassificans*	19
Crustacean, *Porcellio scaber* (Isopoda)	Hepatopancreas	Apparently healthy	PCR	*Rhabdochlamydia porcellionis*	64
Mollusc, *Crassostrea gigas* (Bivalvia)	Ctenidia, mantle	—	LPS	Uncharacterized chlamydia-like	84

[a] To date, only *C. pneumoniae* has been detected in amphibians. Molecular evidence for the presence of novel chlamydial lineages was obtained from different environmental samples, such as soils, marine sediments, freshwater, wastewater, and water conduit systems (reviewed in references 14 and 18).

[b] Note that the urethral and genital tracts open into a urogenital sinus in marsupials, whereas they open into a cloaca in birds and reptiles.

[c] PCR detection targeted mainly ribosomal operon fragments. Isolation was on mammalian cells for *Simkania negevensis* and *Waddlia* species and on amoebal coculture for *Parachlamydiaceae* (see also Tables 2 and 4). LPS indicates that positive reactivity was found using anti-chlamydial LPS antibodies.

[d] Taxa without specific names refer to uncharacterized chlamydiae reputed to belong to these families on the basis of 16S rRNA gene sequences. Novel lineages refer to uncharacterized chlamydiae that are not in any of the described *Chlamydiales* families. Uncharacterized chlamydia-like refers to chlamydia-like organisms for which precise molecular data are lacking.

[e] —, not applicable.

[f] Compared to the chlamydia-free sister-species *Bemisia argentifolii*.

matis, or whether the internalized neochlamydiae may enhance the pathogenicity of the amoebae remains to be elucidated. Its presence within *Acanthamoeba* may also be coincidental, since *Acanthamoeba* keratitis is a well-established clinical entity that occurs especially in patients wearing contact lenses (70).

The role of other *Parachlamydiaceae*, such as *Protochlamydia amoebophila* (11), is still unknown. Some *Parachlamydiaceae* might be involved in bronchitis, atherosclerosis, uveitis, and urogenital infection, since 16S rRNA gene sequences related to *Parachlamydia* were also amplified from mononuclear cells taken from a patient with bronchitis (80), as well as from arterial, aqueous humor, and cervical samples (73).

***Simkania negevensis*.** *Simkania negevensis* is another chlamydia that resists destruction by free-living amoebae and that may use the amoebae as an environmental reservoir (59, 61). Its role as an emerging human respiratory pathogen is suspected (32). Epidemiological studies using PCR, cell culture, and serology have documented not only the worldwide presence of this microorganism (31, 33, 39, 58) but also its association with bronchiolitis in infants (39, 60, 81) and with lower respiratory tract infections in adults (34, 66, 67, 81). More recently, *S. negevensis* DNA has also been amplified from human arterial biopsy specimens (32).

Uncharacterized chlamydia lineages. In addition, various 16S rRNA gene sequences not specifically belonging to any known chlamydia species have been obtained by PCR and sequencing from different human samples, including arteries and aqueous humors (73). It is noteworthy that some of the DNA sequences amplified from aqueous humors of patients suffering from uveitis showed a high level of similarity with the 16S rRNA gene sequences of the arthropod parasite *Rhabdochlamydia* (reviewed in reference 14).

Infections in Other Vertebrates

Molecular approaches have shown that all classes of vertebrates are exposed to chlamydiae (Table 1) (reviewed in reference 14). Further studies are necessary to reliably identify chlamydial species and to clarify the pathogenic potential of each novel chlamydia in animals, since most of these studies are based only on DNA amplification plus sequencing and since most sequenced DNA fragments are less than 300 nucleotides in length (7, 73, 90, 93).

***Parachlamydiaceae*.** *Parachlamydiaceae* could have some importance as ocular pathogens of cats (93), urogenital and ocular pathogens of various species of Australian marsupials (7, 20), and systemic pathogens of reptiles (6, 90). *Neochlamydia* sequences have been obtained from artery samples of swine and gills of fishes (73).

***Simkaniaceae*.** The presence of *Simkania*-related organisms in vertebrates is indicated by PCR studies: Soldati et al. obtained 23S rRNA gene sequences with some similarity to *Simkania* genes from various tissues of reptiles (90), and *Simkania*-like 16S rRNA gene sequences were also obtained from other animal species, but their affiliation to the *Simkaniaceae* clade is not clearly established (73, 80).

***Waddliaceae*.** *Waddlia chondrophila* (85) is a new agent of bovine abortion. This chlamydia has been isolated from two aborted fetuses, in the United States (21) and in Germany (53). A recent serological study revealed a significant statistical association between anti-*Waddlia* antibody titers and cows that have aborted (22). Future research on the mechanisms leading to abortion and on the abortive potential of *Waddlia* are warranted, given the veterinary and socioeconomic impact of abortion in cattle. More importantly, the role of *W. chondrophila* as an emerging agent of bovine abortion should lead to the evaluation of its role as a potential zoonotic agent, since *C. abortus*, another ruminant abortigenic chlamydia, was shown to cause zoonotic abortion in humans (83). The *Waddlia* 16S rRNA gene has also been detected in urogenital samples from healthy Australian marsupials (*Potorous gilbertii*) (7). Moreover, a new species, *Waddlia malaysiensis*, has been isolated from urine samples from the Malaysian fruit bat *Eonycteris spelaea* (9, 10). This may have important epidemiological implications given the important role of bats as vectors of various zoonotic pathogens.

***Piscichlamydia*.** *Piscichlamydia salmonis* is a recently identified chlamydial organism detected in the gill tissues of Atlantic salmon and thus indicated as the probable etiologic agent of the gill epitheliocystis in this fish species (24). Gill epitheliocystis is an infectious disease caused by chlamydia-like organisms, occurring worldwide and affecting several marine and freshwater bony fish species, most of which have important economic value. Limited molecular studies have indicated chlamydiae, not necessarily belonging to the same *Piscichlamydia* lineage, as etiologic agents of this disease (73).

Uncharacterized chlamydia lineages. New 16S rRNA gene phylotypes, presumably representing distinct lineages, have been detected in various sample types from several mammals, birds, and fishes (7, 20, 73).

INTERACTIONS BETWEEN CHLAMYDIAE AND AMOEBAE

Free-living amoebae such as *Acanthamoeba* and *Hartmannella* play a key role as reservoirs (Fig. 1) and/or vectors for a variety of amoeba-resisting microorganisms (reviewed in reference 43), including the chlamydiae. *Acanthamoeba* is a suitable host for most *Parachlamydiaceae* (1, 76) and *S. negevensis* (59, 61), whereas *Hartmannella*, *Balamuthia*, *Dictyostelium*, and *Naegleria* (Table 2) are also able to sustain infection by some *Parachlamydiaceae* (57, 89), *S. negevensis* (75), and *Waddlia chondrophila* (78). Various interactions between chlamydiae and amoebae may take place, depending on the chlamydial and the amoebal species and strain and on environmental conditions. Thus, *P. acanthamoebae* is endosymbiotic at 25 to 30°C and lytic at 32 to 37°C for *Acanthamoeba* spp. (45). Since the temperature of the human nasal mucosa is generally lower (about 30°C) than that of the lower respiratory tract (generally between 35 and 37°C), *P. acanthamoebae* could reside symbiotically within amoebae present on the nasal mucosa and induce the lysis of the amoebae that reach the lower respiratory tract, inducing their own release and possibly starting an infectious process. Conversely, the virulence of the amoebae could be increased when harboring chlamydiae. Fritsche et al. (35) reported that the cytopathic effect on human embryonic tonsillar fibroblasts was enhanced by 66 to 70% when *Acanthamoeba* contained the *Parachlamydia*-related symbiont UWE25, which was recently designated as a new genus and species, *Protochlamydia amoebophila* (11).

Additionally, the growth rate of the amoebae may be differen-

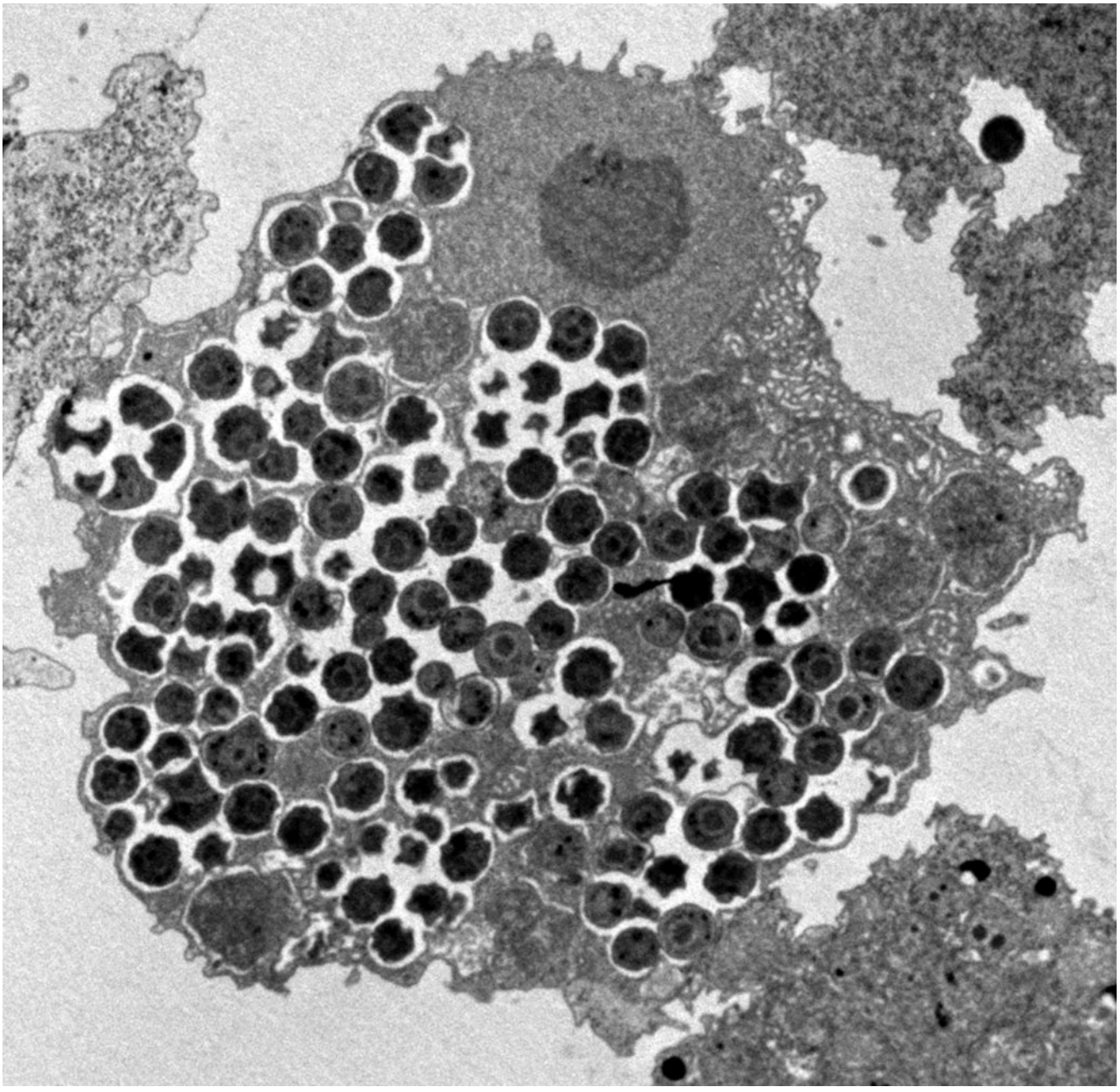

FIG. 1. *Parachlamydia acanthamoebae* strain BN9 within *Acanthamoeba polyphaga*, as seen by electron microscopy. Magnification, ×3,500.

tially affected by their parachlamydial symbionts. Thus, *Hartmannella vermiformis* infected by *Neochlamydia hartmannellae* grew more rapidly, while an *Acanthamoeba* sp. infected by *Protochlamydia amoebophila* (strain UWE25) grew more slowly, with respect to the aposymbiotic amoebae (12). Interestingly, this amoeba-parachlamydia couple (*Acanthamoeba* sp. strain UWC1/*Protochlamydia amoebophila* UWE25) was the same that showed the stronger cytopathic effect enhancement in the study by Fritsche et al. (35). Similarly, amoebal development stages, such as encystment (*Acanthamoeba*), enflagellation (*Naegleria*), or body fruiting (*Dictyostelium*), may be differentially affected depending on the infecting chlamydial strain (57, 75, 76, 78, 89). Notably, only *Hartmannella* strains were susceptible to infection at the first challenge with *Waddlia chondrophila*, whereas *Waddlia* grown in hartmannellae acquired the ability to infect several other free-living amoebae (78).

Whether chlamydiae and amoebae in some way influence each other's virulence deserves additional studies. However, it is likely that the ability of *P. acanthamoebae* to resist the microbicidal effector mechanisms of macrophages (44) has been acquired during their long history of coevolution with amoebae (43).

PROCESSING OF MICROBIOLOGICAL SPECIMENS

Little information is available on the optimal procedures for collecting and processing the novel chlamydiae. Handling of

TABLE 2. Known protist host range for *Chlamydiales*

Chlamydial family and species	Known protist host (known host[s])	Successful exptl infection	Features	Unsuccessful exptl infection	Reference(s)
Parachlamydiaceae					
Parachlamydia acanthamoebae	*Acanthamoeba* sp.	*Acanthamoeba* spp.[a]	Intravacuolar or free in the cytoplasm, cysts do not form or are chlamydia free, a third developmental stage has been described	*Naegleria* sp., *Saccamoeba* sp.	1, 13, 41
Neochlamydia hartmannellae	*Hartmannella vermiformis*	*Hartmannella vermiformis*[a], *Dictyostelium discoideum*[a]	Free in the cytoplasm, increase of amoeba growth rate, developmental cycle not disturbed, release of uninfected spores	*Acanthamoeba* spp.[b], *Comandonia operculata*, *Naegleria* spp., *Willaertia magna*	12, 57
Neochlamydia sp.[c]	*Acanthamoeba* sp.	*Acanthamoeba* sp., *Dictyostelium discoideum*		*Hartmannella vermiformis*	36, 89
Protochlamydia amoebophila	*Acanthamoeba* sp.	*Acanthamoeba* spp.[b], *Dictyostelium discoideum*	Increased cytopathic effect rate on mammalian cells, may decrease amoeba growth rate, cyst may contain chlamydial inclusion	*Acanthamoeba* spp.	11, 12, 35, 36
Other *Parachlamydiaceae*[d]	*Acanthamoeba* sp.	*Acanthamoeba* spp.[b], *Balamuthia mandrillaris*, *Hartmannella vermiformis*, *Dictyostelium discoideum*, *Naegleria* sp., *Willaertia magna*	Continuous excretion of elementary bodies from all but *H. vermiformis*, *Dictyostelium* fruiting body development strongly disturbed	*Acanthamoeba* spp., *Naegleria* spp.	12, 36, 76, 89
Simkaniaceae					
Simkania negevensis	Amoeba? (human)	*Acanthamoeba* sp., *Balamuthia mandrillaris*[f], *Hartmannella vermiformis*[f], *Naegleria clarki*	Survives within cyst and cyst walls, with or without amoebal cytoplasm[e], production of *Simkania*-free cysts	*Acanthamoeba* spp., *Hartmannella vermiformis*[f], *Dictyostelium discoideum*[f], *Naegleria* spp., *Willaertia magna*[f]	32, 59, 61, 75
Fritschea bemisiae	(*Bemisia tabaci*)			*Acanthamoeba*	28
Waddliaceae					
Waddlia chondrophila	(Bovine)	*Hartmannella vermiformis*, *Acanthamoeba* sp.[g], *Dictyostelium discoideum*[g], *Hyperamoeaba*-like[g], *Vahlkampfia ovis*[g], *Naegleria* sp.[g]	Intravacuolar or free in the cytoplasm of *Hartmannella*, successful infection only after passage in *Hartmannella*, encystment inhibited for all but *Vahlkampfia ovis*	*Acanthamoeba* spp., *Balamuthia mandrillaris*, *Echinamoeba* sp., *Vannella miroides*, *Comandonia operculata*, *Naegleria* spp.	78
Chlamydiaceae					
Chlamydophila pneumoniae	(Land vertebrates)	*Acanthamoeba* sp.	Atypical inclusions at 10–14 days p.i., *C. pneumoniae* retains infectivity for HL cells	Not tested	27

[a] Various strains. When 18S rRNA genotyping of *Acanthamoeba* spp. was performed, strains belonged to the T4 lineage.
[b] Various strains. When 18S rRNA genotyping of *Acanthamoeba* spp. was performed, strains belonged also to non-T4 lineages.
[c] *Neochlamydia* strains UWC22 (human corneal sample) and TUME1 (water conduit).
[d] *Acanthamoeba* endosymbiont strains UWE1 and k-cont. These strains could belong to distinct new species or genera within the *Parachlamydiaceae*.
[e] Not in all strains tested.
[f] *Acanthamoeba*-adapted *Simkania*.
[g] *Hartmannella*-adapted *Waddlia*.

clinical samples for detection of chlamydiae should be performed in a biosafety level 2 security laboratory, given their pathogenic potential and the fact that various chlamydiae share the same ecological niche (the amoebae) with other amoeba-resisting bacteria of established pathogenicity, such as *Legionella pneumophila*.

The obligate intracellular nature of chlamydiae implies that an adequate specimen must contain infected host cells and/or a sufficient amount of extracellular elementary bodies. Since extracellular elementary bodies are unlikely to be present during persistent infection, the presence in the specimen of infected host cells is mandatory for both culture and nucleic acid amplification. For mucosal specimens, swabbing seems appropriate for the recovery of a sufficient amount of infected cells. Alginate swabs should be avoided since they may produce artifacts when Giemsa staining is performed. Cotton swabs are preferred. Sampling error may be reduced by scraping of mucosal surfaces, which increases the cellular yield compared to simple swabbing. However, this somewhat invasive procedure may induce bleeding and should be used only for selected indications.

Specific transport media such as the 2-sucrose phosphate or the sucrose-glutamate phosphate medium, originally developed for rickettsiae, may be used. Commonly available viral transport media generally contain antimicrobial agents and should therefore be avoided, since macrolides and tetracyclines inhibit the growth of both *Parachlamydia* (72) and *Simkania* (32) and tetracyclines are active against *Waddlia* species (10, 85). Bacterial overgrowth may be prevented by adding gentamicin (10 μg/ml) and/or vancomycin (100 μg/ml) to the transport medium. Although amphotericin B (1 to 5 μg/ml) is frequently used to prevent fungal overgrowth in cell culture, this compound should be avoided for amoebal coculture given the susceptibility of most free-living amoebae to amphotericin B.

Parachlamydiaceae, *Simkania negevensis*, and *Waddlia* spp. have been isolated and propagated in cell cultures or amoebae by using transport media and/or culture media containing penicillins (1, 10, 21, 53, 60). However, the use of penicillins should ideally be avoided given the susceptibility of *Chlamydiaceae* and the potential susceptibility of the other chlamydiae to penicillins and, by analogy with what is known for *Chlamydiaceae*, given the risk of induction of persistent nonmultiplying aberrant forms.

Microbiological specimens should be stored at 4 to 8°C and processed as soon as possible. If the time between collection and processing is >24 h, specimens should be frozen at −70°C. Freezing may result in a loss of chlamydial viability. This loss has been estimated at up to 20% for *Chlamydiaceae* and may be reduced by the addition of fetal bovine serum (2 to 10%) (2). No data are available for novel chlamydiae.

NUCLEIC ACID AMPLIFICATION

Nucleic acid amplification by PCR and related techniques is widely used in clinical microbiology laboratories in order to detect and/or identify any microorganisms that may be present in clinical samples. These molecular techniques are especially useful in epidemiological studies and as diagnostic tools, e.g., when used for the identification of microorganisms that are difficult to grow in culture. Additionally, PCR approaches that target phylogenetically informative genes such as the 16S rRNA gene contribute to the identification of new strains and species.

PCR techniques that target mainly the ribosomal operon have recently been developed to study the novel chlamydiae. However, sequences of other genes of additional diagnostic and phylogenetic value, such as the ATP/ADP translocase-encoding gene (42), are becoming available. At present, whole genome sequences are available for various *Chlamydia* and *Chlamydophila* strains, for *Protochlamydia amoebophila* strain UWE25 (55), and for *S. negevensis* (see the website http://www.tigr.org/tdb/mdb/mdbinprogress.html), whereas a 16.6-kbp genome portion, including the ribosomal operon and nine other genes, has been determined for *Fritschea bemisiae* (92). PCR tests that have been developed mainly for diagnostic purposes are summarized in the following section.

Species and Strain Identification

Species and strain identification may be achieved by (i) applying taxon-specific primers, either directly or as inner primers in a nested PCR; (ii) analysis of electrophoretic profiles after enzymatic restriction of amplicons (PCR-restriction fragment length polymorphism); or (iii) sequencing of PCR products. For the new chlamydiae, sequencing of the 16S rRNA gene remains the optimal method to infer strain affiliation, since this housekeeping gene carries some phylogenetic information and since 16S rRNA gene sequences are available for all type strains. Supporting this idea is the recent study of Maraha et al. (69), which, after sequencing of various 16S rRNA gene clones obtained with primers considered specific for *C. pneumoniae*, found that they were parachlamydiae.

The use of a primer set amplifying a small portion of the gene (e.g., 300 bp) may be ideal to screen a large numbers of samples, mainly due to the reduced cost of this approach. This approach may also be useful in the setting of degradation of nucleic acids, as in the case of paraffin-embedded tissues (6, 90). However, sequencing of larger gene portions or of other less conserved genes provides phylogenetic information that is necessary to reliably infer the phylogenetic relationship of the strain.

16S rRNA Genes

Universal/eubacterial primer sets have been used to amplify and sequence the 16S rRNA genes of uncharacterized chlamydia-like organisms first documented by morphological analyses and then described as *Parachlamydiaceae* (5, 36), as *Waddlia chondrophila* (85), and as *Rhabdochlamydia porcellionis* (64). These primer sets have in some instances also allowed identification of new chlamydial phylotypes from whole DNA extracts of environmental samples. However, their application to clinical samples is limited, as DNA from almost all bacteria, including normal microbiota, may be amplified.

Using the growing number of *Chlamydiales* sequences available, pan-chlamydia primer sets have been designed and applied to clinical and environmental samples to specifically detect chlamydiae (Table 3). Thus, using chlamydia-specific primers (For2/Rev2 [Table 3]), Meijer and Ossewaarde (73, 80) amplified 98 chlamydial 16S rRNA gene phylotypes, called

TABLE 3. Primer sequences and specificities

Gene and primer set	Primer sequence (5′→3′)[a]	Amplicon (bp)[b]	Specificity[d]	Reference
16S rRNA gene				
16SIGF	CGG CGT GGA TGA GGC AT	298	*Chlamydiales* 5′ 16S rRNA gene	29
16SIGR	TCA GTC CCA GTG TTG GC			
ccF	CCT CGG GTT GTA AAG CAC TTT CGC	512	*Chlamydiales*[f]	61
ccR	CCC CGT CAA TTC TTT TGA GTT T			
CF1	CGT GGA TGA RGC ATG CRA GTC G	1,445	*Chlamydiales*	17
CR6	GTC ATC RGC CYY ACC TTD SRC RYY TCT			
FOR2	CGT GGA TGA GGC ATG CAA GTC GA	264	*Chlamydiales*	80
REV2	CAA TCT CTC AAT CCG CCT AGA CGT CTT AG			
ZpF	AAA GGT AAC GAA TAA TTG CCT	405	*S. negevensis*	60
ZpR	GCA CAG TCG GGG TTG AGA CCG ACT			
23S rRNA gene				
23SAPF2	GAA CCT GAA ACC ART AGC	92	*Chlamydiales*	90
23SAPR	CCT TTT GCA TGA TGA GCC AG			
AF[c]	CAC AGG TAG GCA TGA TGA	1,099	*S. negevensis*	30
BR	CTA GCT GCG GGT AAA CG			
IntF	TTA GAT GCA CAA TGG ATA GTT GGA	338	*S. negevensis* intron I	30
IntR	CCA TCA GCG CTC ATG TGC TCA			
Other genes				
TS1F	ATG CTT TCG TTC TGG TCT AC	180	*S. negevensis* Hsp60 gene	81
TS1R	CCT GCA CGG AGA CGG TTG AC			
Adp81F[e]	TAG TGA TCT GCT ACG GGA TTT	81	*P. acanthamoebae* ATP/ADP translocase gene	51
Adp84R	TTG GAT TAG GAT ATT GCT TAA A			
Adp_probe	6-FAM-5′-AACCTTGTAGAAGTAACCTGGAAGAACCAGC-3′-TAMRA			

[a] For degenerate primers, D is A, G, or C; R is A or G; S is C or G; and Y is T or C.
[b] Lengths of amplicons may vary slightly depending on the chlamydia taxon.
[c] The IntF/IntR set used in nested PCR on AF/AR amplicons. There are four mismatches with the *F. bemisiae* sequence for primers AF and IntF but no mismatch for primer AR. There are three to seven mismatches with sequences of other *Chlamydiales* for primers AF and AR.
[d] Specificity was determined with the sequences available at the time of primer design. For pan-chlamydia primers, such specificity may not cover all the newly determined sequences.
[e] This primer set was applied in a real-time PCR with an internal probe.
[f] Primer ccF matches exactly the last seven nucleotides at the 3′ end but has two to five mismatches at the 5′ end for sequences other than *S. negevensis*.

CRG1 to CRG98. Other authors (7, 20, 93) have applied the chlamydial primer set 16SIGF/16SIGR described by Everett et al. (29) to amplify nearly the same region at the 5′ end of the 16S rRNA gene from clinical samples from various animals. Applying this primer set to koala samples, Devereaux et al. (20) determined various chlamydial sequences, named UKC (for uncultured koala chlamydia), and they noted that several of these sequences have a C or a T at primer position 9 (position 58 in *P. acanthamoebae* 16S rRNA gene numbering; accession no. Y07556). The same is true for several environmental clones sequenced by Horn and Wagner (56). Molecular studies that used the pan-chlamydia forward primer described by Ossewaarde and Meijer (80), ending at that position but with an A, may well have missed several chlamydial genotypes (20). The primers ccF and ccR, developed by Kahane et al. (61) to detect *Simkania*, amplify a 512-bp fragment from position 420 to 930 of the 16S rRNA gene and may also be considered pan-chlamydia primers. The degenerate primers CF1/CR6 have been used to amplify nearly complete 16S rRNA genes from all known chlamydiae (17). Species-specific primers targeting the 16S rRNA gene have also been designed. Kahane et al. developed an *S. negevensis*-specific PCR by using the primer set ZPF/ZPR, which amplifies a 398-bp fragment of the 16S rRNA gene (position 457 to 859 in the *S. negevensis* numbering; accession no. U68460) (39, 60). ZPF/ZPR may also be applied as an inner set in a nested PCR, using ccF/ccR as external primers (61).

23S rRNA Genes

Recently, Soldati et al. (90) designed a pan-chlamydia primer set, 23SAPF2/23SAPR, amplifying a 92-bp fragment of the 23S rRNA gene. This primer set, applied to 90 tissue samples from various species of reptiles suffering from various granulomatous diseases, successfully amplified *C. pneumoniae* DNA (n = 9; 10%) and DNAs of *Parachlamydia*/*Simkania*-related organisms (n = 49; 54.4%).

S. negevensis and *F. bemisiae* 23S rRNA genes contain a group I intron (30, 92). Everett et al. (30) designed *Simkania*-specific primer sets in order to amplify the region of the gene harboring this intron. A first primer set, AF/BR, amplifies a 1,100-bp region of the 23S rRNA gene flanking the intron, whereas an inner primer set, IntF/IntR, targeting the intron sequence, produces a 338-bp fragment (Table 3). During a first amplification, this nested PCR strategy might also detect intronless strains (yet unrecognized). It has been applied to detect *S. negevensis* from water samples (61). This nested PCR, however, did not detect *F. bemisiae*. Indeed, while complete overlapping for the AR primer exists for sequences of both *Fritschea* species, the *F. bemisiae* sequence has four nucleotide mismatches for the AF and IntF primers and a 16-bp nonoverlapping fragment for the IntR primer. Three to seven nucleotide mismatches are present for the AF/BR set with sequences of other *Chlamydiales*.

Other Genes

Other genes have been used as targets for amplification by PCR. Petrich et al. (81) developed a *Simkania negevensis*-specific PCR targeting the heat shock protein 60-encoding gene (*groEL*). This PCR was successfully applied both to peripheral blood mononuclear cells taken from patients with chronic obstructive pulmonary disease and to nasopharyngeal swabs from infants with respiratory illness and nursing home patients. Greub et al. used a real-time PCR targeting the *tlc* gene for the diagnosis of *P. acanthamoebae* infection (51). This gene encodes an energy parasite enzyme, the ADP/ATP translocase, which is present only in *Rickettsiales*, *Chlamydiales*, and plant plastids (42). Since the gene is not present in other bacterial genomes, the risk of false-positive PCRs due to amplification of DNAs of other bacterial species such as *Neisseriaceae*, streptococci, and other oropharyngeal colonizers that frequently contaminate respiratory samples is limited. The specificity of this PCR was increased by using a real-time system with a specific primer set and a specific probe. An additional advantage of this real-time PCR was its reliability in quantifying the number of *P. acanthamoebae* organisms (45, 49). However, the high specificity of this PCR prevents its use to detect *Neochlamydia* and any infection due to more distant members of the *Chlamydiales*.

Recently Griffiths et al. (51a) described insertion/deletion signatures, i.e., indels, characteristic for *Chlamydiales*, including *Chlamydiaceae* and *Parachlamydia*, *Neochlamydia*, *Simkania*, and *Waddlia* species. These indels are within five essential proteins: the RNA polymerase alpha subunit (RpoA), the elongation factors Tu and P (EF-Tu and EF-P), the DNA gyrase beta subunit (GyrB), and the lysyl-tRNA synthetase (LysRS). In addition to providing very interesting phylogenetic data, this work will probably have some practical applications also, as it describes new tools for the identification of chlamydiae beside the ribosomal operon.

False-Positive and False-Negative Results

A major problem of PCR-based diagnostic approaches is the risk of false-positive results. These are principally due to (i) vertical contamination of the sample by previously amplified products, (ii) the accidental presence of the nucleic acid target in the specimen, and (iii) unexpected low specificity of the PCR protocol.

Vertical contamination is especially frequent when a high number of reactions that target the same DNA region are carried out or when nested PCR is performed. Detection of vertical contamination may be facilitated by using multiple blank reaction tubes intermixed with sample reaction tubes and by confirming any positive results by a second PCR that targets a second gene (47). The risk of vertical contamination may be reduced by performing the extraction, amplification, and postamplification steps in separate rooms; by using hot-start polymerases; and by decontaminating workplaces (e.g., by UV irradiation and PCR reagents such as restriction enzyme and/or dUTP-uracil-DNA glycosylase) at regular time intervals.

False-positive amplification products may also result from the presence of the microorganism (or of part of its genomic material) in PCR reagents (water, deoxynucleoside triphosphates, or enzyme stocks). The presence of the target DNA in laboratory reagents has caused false-positive results for chlamydiae in at least two studies (69, 73). False-positive results may also occur due to the presence of parachlamydiae in the noses of healthy volunteers (1) and the presumable secondary contamination of lower respiratory tract samples with the subjects' oropharyngeal secretions.

An unexpectedly low specificity of the PCR protocol may be attributable to the low specificity of the primers used because of the presence in the specimen of as-yet-unrecognized species harboring the same target sequence (69) or may originate from suboptimal temperatures and magnesium concentrations.

Given the significant risk of false-positive results, a single positive PCR or nested PCR is only a first indirect hint of the presence of a chlamydia in the specimen. Confirmation of the presence of viable microorganisms by reverse transcription-PCR or culture may be important. On the other hand, false-negative results may result from inadequate sensitivity of the PCR protocol and from the presence of PCR inhibitors.

CELL CULTURE

The isolation and propagation of intracellular bacteria such as chlamydiae in a cell culture system are fundamental in demonstrating the presence of a viable organism in the clinical sample and characterizing the strain beyond its gene sequence. Considering the huge diversity within chlamydiae that is suggested by molecular studies, efforts should be made to adapt cell culture protocols to allow the recovery of these new species. *Chlamydiaceae* have been cultivated into embryonated eggs, but this culture system was relatively rapidly replaced by cell cultures, which are easier and more sensitive (2). Embryonated eggs are used today mainly for massive production of antigens or for propagation of fastidious strains (2). Egg culture has not yet been tested for any novel chlamydia.

The possibility of *Chlamydiaceae* entering into a persistent/cryptic state is a key pathogenic feature explaining the wide range of chronic diseases that most *Chlamydiaceae* seem to induce in their vertebrate hosts (3, 54). However, persistence complicates the laboratory diagnosis of chlamydial infection, at least when using culture-based approaches, potentially leading to false-negative results. It is yet unknown whether *Parachlamydiaceae*, *Simkaniaceae*, and *Waddliaceae* may also show a persistent state and

TABLE 4. Mammalian cell culture of *Chlamydiales* species

Chlamydial family and species	Known host(s)	First isolation	Successful propagation in mammalian cells	Reference(s)
Chlamydiaceae				
Chlamydia spp.	Mammals	Embryonated eggs	Various cell lines[a]	2
Chlamydophila spp.[h]	Land vertebrates	Embryonated eggs	Various cell lines[a]	2
Parachlamydiaceae				
Parachlamydia acanthamoebae	*Acanthamoeba* sp.	*Acanthamoeba* spp.[b]	Vero,[c] HeLa, NCI-H292, blood-derived human macrophages[d]	1, 13, 44, 49, 72
Protochlamydia amoebophila	*Acanthamoeba* sp.	*Acanthamoeba* spp.	None	11
Neochlamydia hartmannellae	*Hartmannella vermiformis*	*Hartmannella vermiformis*	None	57
Simkaniaceae				
Simkania negevensis	Human, amoeba	Vero[e]	Vero, HEp-2, human U937, macrophages, Buffalo green monkey cells	32, 60, 61, 75
Fritschea spp.	Homopteran insects		None	28, 92
Waddliaceae				
Waddlia chondrophila[h]	Bovine	Bovine turbinate cells[f]	Bovine turbinate cells, McCoy cells, Buffalo green monkey cells, human diploid fibroblasts, murine P388D1 macrophages	21, 53
Waddlia malaysiensis	Fruit bat	Vero[g]	Vero, MRC-5, A549, HEK, HEp-2, B-lymphoblastoid cell line, LLC-MK2, 3T3, BHK	9, 10
Rhabdochlamydiaceae				
Rhabdochlamydia spp.	Arthropods		None	19, 64
Piscichlamydia salmonis	Salmonid fish		None	24

[a] Penicillin sensitive.
[b] Different strains have been used (see Table 2). Sensitive to aminoglycosides in *Acanthamoeba* coculture (72).
[c] Other authors reported on the failed propagation on this cell line, as well as on McCoy cells, murine P388D1 macrophages, and human embryonic lung fibroblasts (72).
[d] Rapid induction of apoptosis (49).
[e] Longer life cycle (about 2 weeks).
[f] Vacuolization at 24 to 48 h p.i.; periodic acid-Schiff stain negative.
[g] Cytopathic effect 5 to 7 days p.i.; periodic acid-Schiff stain positive.
[h] *Chlamydophila pneumoniae* and *Waddlia chondrophila* have been cultivated experimentally in *Acanthamoeba* and various amoeba species, respectively (see Table 2).

which cell lines or amoebal strains may especially help in resuscitating such viable but nonculturable bacteria.

Mammalian Cells

As obligate intracellular parasites, chlamydiae are generally isolated and grown on monolayers of mammalian cells. To screen for the presence of bacteria in cell culture, standard stains such as Giemsa and Gimenez stains can be used. Immunofluorescence techniques, using homemade specific antibodies raised against each novel chlamydia, are employed in some laboratories.

Chlamydiaceae. Various cell lines are routinely used for members of the *Chlamydiaceae*, with each species or biovar showing a relative specificity for a given cell type (2, 86). Following inoculation, centrifugation (1,000 to 3,000 × *g*, 1 h, 30 to 35°C) is generally used for *Chlamydiaceae*, as it increases the infectious rates by about 100- to 1,000-fold (79, 86). The culture is then incubated for 5 to 14 days, with some protocols requiring more than one passage. Penicillin and derivatives should be avoided because they may disturb the development of *Chlamydiaceae* by inducing aberrant nonreplicating developmental stages (3). Bacterial and fungal overgrowth is prevented by adding gentamicin (10 to 50 μg/ml), vancomycin (100 μg/ml), and amphotericin B (1 to 4 μg/ml). Since chlamydiae are "energy parasites" that utilize the host cell ATP pool (42, 79), inhibitors of eukaryotic metabolism such as cycloheximide (1 to 5 μg/ml) may be added to the growth medium. This favors chlamydial growth by limiting host cell utilization of energy. The importance of cycloheximide to promote the growth of *Chlamydiaceae* in cell culture is for some *Chlamydiaceae* much smaller than that of the initial centrifugation (86).

Simkania negevensis. *S. negevensis* and *Waddlia* spp. have been grown in various cell lines (Table 4). *S. negevensis* was discovered as a cell culture contaminant (62). Vero cells are currently used to isolate *S. negevensis* from clinical samples (39, 60). However, successful culture has also been obtained using HeLa cells, HEp-2 cells, and the human macrophage cell line U937 (32, 62). To optimize the propagation of *S. negevensis* in Vero cells, Yamaguchi et al. (95) recently applied various protocols that included (i) centrifugation or sonication of the inoculum, (ii) pretreatment of the cell monolayer with polyethylene glycol or DEAE-dextran, and (iii) use of culture media of different compositions regarding fetal calf serum con-

centration and the addition of antibiotics (streptomycin and vancomycin, 100 μg/ml each) and cycloheximide (1 μg/ml). Those authors found that the number of inclusions was significantly higher when the inoculum was centrifuged (1,500 × *g*, 60 min, 35°C). Sonication had no effect. By contrast, pretreatment of Vero cells with either polyethylene glycol or DEAE-dextran decreased the number of inclusions, as did the addition of cycloheximide. Thus, ideal conditions may include centrifugation of the inoculum on untreated Vero cells grown in RPMI 1640 medium with 10% fetal calf serum, the addition of antibiotics, and no cycloheximide. The efficiency of this protocol when applied to clinical samples remains to be evaluated and to be compared to amoebal coculture (see below).

***Waddliaceae*.** *Waddlia chondrophila* (strain WSU 86-1044) was first isolated from a fetal homogenized lung-liver pool by inoculating bovine turbinate cells incubated at 35°C in a penicillin-containing medium without centrifugation (21). The same strain was also propagated in P388D1 mouse macrophages without antibiotics in order to study its developmental cycle (63). Henning et al. (53) reported the isolation of an additional strain (2032/99) from bovine fetal heart on Buffalo green monkey cells, McCoy cells, and human diploid fibroblasts. *Waddlia malaysiensis* was isolated on Vero cells inoculated with pooled urine samples from Malaysian fruit bats, and the strain was successfully propagated in various human, simian, and rodent cell lines (9, 10) (Table 4).

A rapid cytopathic effect on bovine turbinate cells and Vero cells appeared at 36 h postinfection (p.i.) and 5 to 7 days p.i. for *W. chondrophila* (21) and *W. malaysiensis* (10), respectively. Thus, although a large number of cell lines may be appropriate for the growth of *Waddlia* and *Simkania*, Vero cells might be the preferred mammalian cell line, being permissive to representatives of both genera.

***Parachlamydiaceae*.** Mammalian cells are generally not used to grow *Parachlamydiaceae*. A first report on the culture of *P. acanthamoebae* (strain Berg17) in Vero cells (1, 77) has not been confirmed using another *P. acanthamoebae* strain (Bn9) (72). McCoy cells, P388D1 macrophages, and human embryonic fibroblasts were also not permissive to that strain (72). Successful infection of blood-derived human macrophages has, however, been obtained for another *P. acanthamoebae* strain (Hall's coccus) by Greub et al. (49). *P. acanthamoebae* strain UV-7 was propagated in Vero, HeLa, and H292 cells after an initial recovery in amoebal coculture (13). The *Parachlamydiaceae* strain CorvenA4, present in a human bronchoalveolar lavage sample, has been characterized by the 16S rRNA gene sequence only, since its isolation on both Vero and HeLa cells failed (15). Because Maurin et al. (72) found *P. acanthamoebae* susceptible to gentamicin in an *Acanthamoeba* system, the use of aminoglycosides should probably be discouraged since it might hamper the recovery of *Parachlamydiaceae* and some other new chlamydiae.

Other novel chlamydiae. Chlamydiae that infect arthropods, i.e., *Fritschea* and *Rhabdochlamydia*, have not yet been tested for their ability to grow in vertebrate cells, and the vast majority of studies describing other novel chlamydiae have been limited to molecular approaches. Bodetti et al. (7) used HEp-2 cells to isolate chlamydiae from various Australian marsupial species that were swabbed at the urogenital sinus, conjunctiva, and nasopharynx. Inocula were centrifuged at 800 × *g* on cycloheximide-treated cells, and repeated centrifugations at 4 to 7 days p.i. were performed. By using a commercially available anti-chlamydial lipopolysaccharide (LPS) fluorescent monoclonal antibody, six isolates were detected, but only one was successfully subcultured. The specificity of anti-LPS monoclonal antibodies to *Chlamydiaceae* species (but see "Staining" below) and the unknown permissivity of HEp-2 cells to most other chlamydiae (*S. negevensis* is the only documented exception [32]) could explain the low rate of positive results obtained by standard cell culture protocols.

Amoebal Coculture

Free-living amoebae, mainly *Acanthamoeba* spp., have been shown to be natural hosts for several amoeba-resisting bacteria, including *Parachlamydiaceae.* These unicellular eukaryotes have also been successfully used in a cell culture system to isolate a variety of amoeba-resisting bacteria from clinical samples (43, 46).

The fact that *C. pneumoniae* (27) and *S. negevensis* (59, 75) are able to infect *Acanthamoeba* in vitro and the fact that free-living amoebae likely play a role as an environmental host of *Simkania* (61) support the use of *Acanthamoeba* to isolate novel chlamydiae from clinical samples. Of note, *Neochlamydia hartmannellae* (57) (but not other *Neochlamydia* spp. [36]), has been successfully grown within *Hartmannella vermiformis* but not within *Acanthamoeba*. Moreover, *Waddlia chondrophila* may grow in *Acanthamoeba* and other free-living amoebae, but only after subcultivation in *H. vermiformis* (78). Efforts to cultivate *Fritschea* in *Acanthamoeba* have failed (28). In addition, experimental studies have shown that (i) a wide diversity of free-living amoebae are able to sustain the growth of the new chlamydiae and (ii) not all strains of a free-living amoeba species are susceptible to infection by these agents. This should be taken into account when interpreting negative coculture results (Table 2) (35, 57, 75–78, 89). Thus, the use of more than one strain of *Acanthamoeba* and/or the use of several amoeba species (e.g., *Acanthamoeba* and *Hartmannella*) is recommended to increase the rate of isolation of chlamydiae, at least from clinical samples that give a positive PCR result. In addition to the possible problem of a persistent viable but noncultivable stage, false-negative culture may also occur due to the use of a cell that is not permissive to a given species (i.e., *Neochlamydia* and *Acanthamoeba*) or due to the presence of antibiotics in the amoebal coculture to which the bacterium is susceptible. False-positive results might occur if the cell line is contaminated with an endosymbiotic chlamydia (which will not be easily detected) or if the broth is not adequately filtered and/or heat sterilized.

Amoebal Enrichment

Since amoebae may harbor *Parachlamydiaceae*, *S. negevensis* (59, 61, 75), and *Waddlia chondrophila* (78), it may be useful to first isolate amoebae from clinical samples and then look within these amoebae for whether a chlamydia is present. Screening of environmental samples for the presence of amoebae may also contribute to the identification of a possible source of infection (e.g., humidifiers or air conditioning systems).

Amoebal enrichment may be performed using established pro-

tocols (43, 46, 87). Specimens are generally seeded on nonnutrient agar preinoculated with heat-inactivated *Escherichia coli*.

Stainings

Detection of bacteria that grow in both cell culture and amoebal culture may be performed using different staining methods (Gram, Giemsa, Gimenez, or immunofluorescence) or using fluorescent in situ hybridization (FISH). Since chlamydiae are inconsistently stained with Gram stain, alternative stains are preferred, such as Giemsa and Gimenez stains. With Giemsa stain, the bacteria stain blue. To obtain a better contrast, alternative staining approaches such as the Giemsa-May-Grünwald stains may be useful, since the cell nucleus is stained with eosin, whereas the chlamydiae appear as clear blue dots on the slightly stained cytoplasm background. This staining method, slightly modified to use eosin, a thiazine derivative, and a fixative methanol solution, is commercially available (Diff-Quick set; Dade, Düdingen, Switzerland) and may be easily implemented even in routine diagnostic laboratory procedures. However, the best staining for screening is probably the Gimenez method (38). The chlamydiae appear as reddish-staining bacteria (fuchsin) against a green background (malachite green). Briefly, the staining solution, which contains basic fuchsin (10 g/liter) in ethanol (10%) and phenol (1%), is diluted in a phosphate buffer (NaH_2PO_4-Na_2HPO_4) and applied to the sample for 1 or 2 min. Then, after washing with water, counterstaining is performed twice with malachite green (0.8% in water) for 15 to 30 seconds. After washing with tap water and dry blotting, the sample is ready to be examined.

The sensitivity of immunofluorescence for chlamydia detection is probably higher than that of standard staining techniques. However, its use for the detection of novel chlamydiae is limited by the specificity of anti-*Chlamydiaceae* antibodies (anti-LPS or anti-major outer membrane protein) and by the limited availability of antibodies directed against the new chlamydia species. It should be noted that reactivity against anti-*Chlamydiaceae* LPS antibodies has been reported for the distantly related fish pathogen *Piscichlamydia salmonis* (24) and for at least two uncharacterized chlamydia-like organisms infecting a fish (52) and an oyster (84).

FISH using rRNA-targeted oligonucleotide probes is widely used for the detection of uncultured bacteria in complex environmental microbial communities. It may also be a valuable tool for the rapid detection of novel chlamydiae in clinical specimens. Poppert et al. (82) developed a set of probes that may specifically detect and differentiate most *Chlamydiaceae* that are pathogenic to humans (*C. pneumoniae*, *C. trachomatis*, and *C. psittaci*) and a "*Chlamydiales*" probe that detects some *Chlamydiaceae* and *Parachlamydiaceae* (82). FISH-stained chlamydiae were demonstrable as early as 12 h p.i., and rRNA highlighted by FISH colocalized with chlamydial antigen, further supporting the validity of this approach. Its use on clinical specimens, however, has not yet been validated.

SEROLOGY

In addition to molecular approaches and culture, serology is increasingly used to assess the pathogenic roles of the novel chlamydiae, even though serology provides only an indirect hint of pathogenicity (5, 31, 48, 66, 71).

An enzyme-linked immunosorbent assay (ELISA) has been developed for the diagnosis of *Simkania negevensis* infection (60, 67). This ELISA, which may detect immunoglobulin A (IgA) and IgG antibodies (but not IgM), was initially designed to work with sera diluted 1:30 to 1:100 for IgA and IgG, respectively (67). With this ELISA, the presence of IgA was empirically considered as an indicator of recent infection, whereas that of IgG suggested recent or past infection. Using this ELISA at the mentioned serum dilutions, as many as 39 to 68% of healthy adults living in different parts of the world (Israel, Canada, the United States, and Denmark) were found to be seropositive (32). Of note, using immunofluorescence and a cutoff for positivity of as low as 1:8, Yamaguchi et al. did not find such a high prevalence, with a 7.5% seroprevalence in adults and a maximum seropositivity of 15% in elderly subjects (94). Thus, the clinical significance of antibody reactivity against *Simkania* should be interpreted with caution, especially when using ELISA. Yamaguchi et al. also showed limited cross-reactivity between *Simkania* and *C. pneumoniae* (94).

Immunofluorescence, which is considered the gold standard for *Chlamydiaceae* (23), has been used for *Parachlamydiaceae* in different studies, supporting the role of these organisms in community-acquired pneumonia (4, 5, 71) and in aspiration pneumonia (48). Whole heat-inactivated bacteria were used as the antigen. Cutoffs for seropositivity used in these studies ranged from an antibody titer of >1:50 (71) to ≥1:128 (4).

To use the same dilutions as those used for the diagnosis of *Chlamydia*-related infections (23), it seems preferable in the future to use serial dilutions starting at 1:8 and not at 1:25 for the diagnosis of infections due to the new chlamydiae. Since among 100 sera taken from healthy blood donors, none exhibited an antibody titer of ≥1:50, we suggest considering an anti-IgG titer of ≥1/64 as evidence of prior infection. Based on our own experience and the criteria used by Marrie et al. (71), we suggest considering the following serological results as indicative of a recent infection: (i) a single IgM titer of ≥1:32, (ii) seroconversion (IgG increase from 0 to ≥1:64 in paired specimens), and (iii) a ≥4-fold rise in the IgG titer between acute- and convalescent-phase sera. Since no definite cutoffs have been established for immunofluorescence for *Simkania* and *Waddlia* spp., we propose to also use these cutoffs for these chlamydiae.

Confirmation of positive immunofluorescence results by Western blotting has been reported for sera that tested positive for *Parachlamydia* (48) and for *Simkania* (94). For *Parachlamydia*, proteins of approximately 60 kDa and 48 kDa were shown to exhibit the strongest antigenicity, whereas for *Simkania*, a strong and specific signal was observed at 64 kDa. More important, this 64-kDa signal was shown to be specific for *Simkania*, still being present after serum adsorption with *C. pneumoniae*. The degrees of cross-reactivity between the different novel chlamydia species and between every member of the *Chlamydiales* remain to be determined. Indeed, false-positive results may be caused mainly by serological cross-reactivities. Therefore, Western blotting and cross-adsorption, which may be of some help in confirming the specificity of any seropositive test result, should ideally be performed.

CONCLUSION

In conclusion, there is increasing evidence of the pathogenicity of *Simkania negevensis* (32) and *Parachlamydia acanthamoebae* (40), especially as agents of lower respiratory tract infection. The pathogenic potentials of additional novel chlamydiae remain to be defined (14). Consequently, there is an urgent need for efficient and standardized diagnostic approaches. Based on the published reports, a combined diagnostic strategy using both molecular approaches and serology as an initial diagnostic screening work-up seems appropriate. Positive results may then be confirmed by cell and amoebal coculture. Future diagnostic challenges include the identification of the best PCR protocols, confirmation of the serological cutoff levels that we have proposed here, and the determination of the optimal growth conditions for each novel chlamydia. We hope that the present review will help stimulate clinical diagnostic microbiology laboratory and research teams to work in this exciting emerging field.

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REFERENCES

1. **Amann, R. I., N. Springer, W. Schönhuber, W. Ludwig, E. N. Schmid, K. D. Müller, and R. Michel.** 1997. Obligate intracellular bacterial parasites of acanthamoebae related to *Chlamydia* spp. Appl. Environ. Microbiol. **63:**115–121.
2. **Barnes, R. C.** 1989. Laboratory diagnosis of human chlamydial infections. Clin. Microbiol. Rev. **2:**119–136.
3. **Beatty, W. L., R. P. Morrison, and G. I. Byrne.** 1994. Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. Microbiol. Rev. **58:**686–699.
4. **Benson, R. F., W. J. Drozanski, T. J. Rowbotham, I. Bialkowska, D. Losos, J. C. Butler, H. B. Lipman, J. F. Plouffe, and B. S. Fields.** 1995. Serologic evidence of infection with 9 *Legionella*-like amoebal pathogens in pneumonia patients, abstr. C-200. Abstr. 95th Gen. Meet. Am. Soc. Microbiol. 1995. American Society for Microbiology, Washington, D.C.
5. **Birtles, R. J., T. J. Rowbotham, C. Storey, T. J. Marrie, and D. Raoult.** 1997. *Chlamydia*-like obligate parasite of free-living amoebae. Lancet **349:**925–926.
6. **Bodetti, T. J., E. Jacobson, C. Wan, L. Hafner, A. Pospischil, K. Rose, and P. Timms.** 2002. Molecular evidence to support the expansion of the host range of *Chlamydophila pneumoniae* to include reptiles as well as humans, horses, koalas and amphibians. Syst. Appl. Microbiol. **25:**146–152.
7. **Bodetti, T. J., K. Viggers, K. Warren, R. Swan, S. Conaghty, C. Sims, and P. Timms.** 2003. Wide range of *Chlamydiales* types detected in native Australian marsupials. Vet. Microbiol. **96:**177–187.
8. **Boman, J., and M. Hammerschlag.** 2002. *Chlamydia pneumoniae* and atherosclerosis: critical assessment of diagnostic methods and relevance to treatment studies. Clin. Microbiol. Rev. **15:**1–20.
9. **Chua, K. B.** 2003. A novel approach for collecting samples from fruit bats for isolation of infectious agents. Microb. Infect. **5:**487–490.
10. **Chua, K. B., J. E. Corkill, P. S. Hooi, C. Winstanley, and C. A. Hart.** 2005. *Waddlia malaysiensis*: an obligate intracellular bacterium isolated from a fruit bat (*Eonycteris spelaea*). Emerg. Infect. Dis. **11:**271–277.
11. **Collingro, A., E. R. Toenshoff, M. W. Taylor, T. R. Fritsche, M. Wagner, and M. Horn.** 2005. '*Candidatus* Protochlamydia amoebophila,' an endosymbiont of *Acanthamoeba* spp. Int. J. Syst. Evol. Microbiol. **55:**1863–1866.
12. **Collingro, A., J. Walochnik, C. Baranyi, R. Michel, M. Wagner, M. Horn, and H. Aspöck.** 2004. Chlamydial endocytobionts of free-living amoebae differentially affect the growth rate of their hosts. Eur. J. Protistol. **40:**57–60.
13. **Collingro, A., S. Poppert, E. Heinz, S. Schmitz-Esser, A. Essig, M. Schweikert, M. Wagner, and M. Horn.** 2005. Recovery of environmental chlamydia strain from activated sludge by co-cultivation with *Acanthamoeba* sp. Microbiology **151:**301–309.
14. **Corsaro, D., and D. Venditti.** 2004. Emerging chlamydial infection. Crit. Rev. Microbiol. **30:**75–106.
15. **Corsaro, D., D. Venditti, A. Le Faou, P. Guglielmetti, and M. Valassina.** 2001. A new chlamydia-like 16S rDNA sequence from a clinical sample. Microbiology **147:**515–516.
16. **Corsaro, D., D. Venditti, and M. Valassina.** 2002. New chlamydial lineages from freshwater samples. Microbiology **148:**343–344.
17. **Corsaro, D., D. Venditti, and M. Valassina.** 2002. New parachlamydial 16S rDNA phylotypes detected in human clinical samples. Res. Microbiol. **153:**563–567.
18. **Corsaro, D., M. Valassina, and D. Venditti.** 2003. Increasing diversity within chlamydiae. Crit. Rev. Microbiol. **29:**37–78.
19. **Corsaro, D., V. Thomas, G. Goy, D. Venditti, R. Radek, and G. Greub.** *Rhabdochlamydia crassificans*, sp. nov., comb. nov., and intracellular bacterial pathogen of the cockroach *Blatta orientalis* (Insecta: Blattodea), and proposal for *Rhabdochlamydiaceae*, fam. nov., a new family of chlamydiae infecting arthropods. Syst. Appl. Microbiol., in press.
20. **Devereaux, L. N., A. Polkinghorne, A. Meijer, and P. Timms.** 2003. Molecular evidence for novel chlamydial infections in the koala (*Phascolarctos cinereus*). Syst. Appl. Microbiol. **26:**245–253.
21. **Dilbeck, P. M., J. F. Evermann, T. B. Crawford, A. C. S. Ward, C. W. Leathers, C. J. Holland, C. A. Mebus, L. L. Logan, F. R. Rurangirwa, and T. C. McGuire.** 1990. Isolation of a previously undescribed rickettsia from an aborted bovine fetus. J. Clin. Microbiol. **28:**814–816.
22. **Dilbeck-Robertson, P., M. M. McAllister, D. Bradway, and J. F. Evermann.** 2003. Results of a new serologic test suggest an association of *Waddlia chondrophila* with bovine abortion. J. Vet. Diagn. Investig. **15:**568–569.
23. **Dowell, S. F., R. W. Peeling, J. Boman, G. M. Carlone, B. S. Fields, J. Guarner, M. R. Hammerschlag, L. A. Jackson, C.-C. Kuo, M. Maass, T. O. Messmer, D. F. Talkington, M. L. Tondella, and S. R. Zaki.** 2001. Standardizing *Chlamydia pneumoniae* assay: recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada). Clin. Infect. Dis. **33:**492–503.
24. **Draghi, A., II, V. L. Popov, M. M. Kahl, J. B. Stanton, C. C. Brown, G. J. Tsongalis, A. B. West, and S. Frasca, Jr.** 2004. Characterization of "*Candidatus* Piscichlamydia salmonis" (order *Chlamydiales*), a chlamydia-like bacterium associated with epitheliocystis in farmed atlantic salmon (*Salmo salar*). J. Clin. Microbiol. **42:**5286–5297.
25. **Emerson, P. M., R. L. Bailey, O. S. Mahdi, G. E. L. Walraven, and S. W. Lindsay.** 2000. Transmission ecology of the fly *Musca sorbens*, a putative vector of trachoma. Trans. R. Soc. Trop. Med. Hyg. **94:**28–32.
26. **Emerson, P. M., S. W. Lindsay, N. Alexander, M. Bah, S.-M. Dibba, H. B. Faal, K. O. Lowe, K. P. McAdam, A. A. Ratcliffe, G. E. Walraven, and R. L. Bailey.** 2004. Role of flies and provision of latrines in trachoma control: cluster-randomised controlled trial. Lancet **363:**1093–1098.
27. **Essig, A., M. Keinemann, U. Simnacher, and R. Marre.** 1997. Infection of *Acanthamoeba castellanii* by *Chlamydia pneumoniae*. Appl. Environ. Microbiol. **63:**1396–1399.
28. **Everett, K. D. E., M. L. Thao, M. Horn, G. E. Dyszynski, and P. Baumann.** 2005. Novel chlamydiae in whiteflies and scale insects: endosymbionts '*Candidatus* Fritschea bemisiae' strain Falk and '*Candidatus* Fritschea eriococci' strain Elm. Int. J. Syst. Evol. Microbiol. **55:**1581–1587.
29. **Everett, K. D. E., R. M. Bush, and A. A. Andersen.** 1999. Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. Int. J. Syst. Bacteriol. **49:**415–440.
30. **Everett, K. D. E., S. Kahane, R. M. Bush, and M. G. Friedman.** 1999. An unspliced group I intron in 23S rRNA links *Chlamydiales*, chloroplasts, and mitochondria. J. Bacteriol. **181:**4734–4740.
31. **Friedman, M. G., A. Galil, S. Greenberg, and S. Kahane.** 1999. Seroprevalence of IgG antibodies to the chlamydia-like microorganism 'Simkania Z' by ELISA. Epidemiol. Infect. **122:**117–123.
32. **Friedman, M. G., B. Dvoskin, and S. Kahane.** 2003. Infections with the chlamydia-like microorganism *Simkania negevensis*, a possible emerging pathogen. Microb. Infect. **5:**1013–1021.
33. **Friedman, M. G., B. Dvoskin, J. Hartley, D. Greenberg, C.-H. Chiu, M. Hammerschlag, D. Lieberman, P. Roblin, M. Gelling, S. Ben-Dror, and S. Kahane.** 2000. Seropositivity to the novel microorganism *Simkania negevensis* in Israel, North America and Great Britain, p. 324. *In* P. Saikku (ed.), Proceedings of the Fourth Meeting of the European Society for Chlamydia Research, Helsinki, Finland, 20–23 August 2000. Società Editrice Esculapio, Bologna, Italy.
34. **Friedman, M. G., N. Birkebael, B. Dvoskin, P. L. Andersen, S. Kahane, and L. Ostegaard.** 2000. Serologic evidence for infection with *Simkania negevensis* in Denmark and its possible association with chronic cough, p. 258. *In* P. Saikku (ed.), Proceedings of the Fourth Meeting of the European Society for Chlamydia Research, Helsinki, Finland, 20–23 August 2000. Società Editrice Esculapio, Bologna, Italy.
35. **Fritsche, T. R., D. Sobek, and R. K. Gautom.** 1998. Enhancement of *in vitro* cytopathogenicity by *Acanthamoeba* spp. following acquisition of bacterial endosymbionts. FEMS Microbiol. Lett. **166:**231–236.
36. **Fritsche, T. R., M. Horn, M. Wagner, R. P. Herwig, K.-H. Schleifer, and R. K. Gautom.** 2000. Phylogenetic diversity among geographically dispersed *Chlamydiales* endosymbionts recovered from clinical and environmental isolates of *Acanthamoeba* spp. Appl. Environ. Microbiol. **66:**2613–2619.
37. **Fritsche, T. R., R. K. Gautom, S. Seyedirashti, D. L. Bergeron, and T. D.**

Lindquist. 1993. Occurrence of bacterial endosymbionts in *Acanthamoeba* spp. isolated from corneal and environmental specimens and contact lens. J. Clin. Microbiol. **31:**1122–1126.
38. **Gimenez, D. F.** 1964. Staining rickettsiae in yolk-sac cultures. Stain Technol. **39:**135–140.
39. **Greenberg, D., A. Banerji, M. G. Friedman, C.-H. Chiu, and S. Kahane.** 2003. High rate of *Simkania negevensis* among Canadian Inuit infants hospitalized with lower respiratory tract infections. Scand. J. Infect. Dis. **35:**506–508.
40. **Greub, G., and D. Raoult.** 2002. *Parachlamydiaceae*: potential emerging pathogens. Emerg. Infect. Dis. **8:**625–630.
41. **Greub, G., and D. Raoult.** 2002. Crescent bodies of *Parachlamydia acanthamoeba* and its life cycle within *Acanthamoeba polyphaga*: an electron micrograph study. Appl. Environ. Microbiol. **68:**3076–3084.
42. **Greub, G., and D. Raoult.** 2003. History of the ADP/ATP-translocase-encoding gene, a parasitism gene transferred from a *Chlamydiales* ancestor to plants 1 billion years ago. Appl. Environ. Microbiol. **69:**5530–5535.
43. **Greub, G., and D. Raoult.** 2004. Microorganisms resistant to free-living amoebae. Clin. Microbiol. Rev. **17:**413–433.
44. **Greub, G., B. Desnues, D. Raoult, and J.-L. Mege.** 2005. Lack of microbicidal response in human macrophages infected with *Parachlamydia acanthamoebae*. Microb. Infect. **7:**714–719.
45. **Greub, G., B. La Scola, and D. Raoult.** 2003. *Parachlamydia acanthamoebae* is endosymbiotic or lytic for *Acanthamoeba polyphaga* depending on the incubation temperature. Ann. N. Y. Acad. Sci. **990:**628–634.
46. **Greub, G., B. La Scola, and D. Raoult.** 2004. Amoebae-resisting bacteria isolated from human nasal swabs by amoebal coculture. Emerg. Infect. Dis. **10:**470–477.
47. **Greub, G., H. Lepidi, C. Rovery, J. P. Casalta, G. Habib, F. Collard, P. E. Fournier, and D. Raoult.** 2005. Diagnosis of infectious endocarditis in patients undergoing valve surgery. Am. J. Med. **118:**230–238.
48. **Greub, G., I. Boyadjiev, B. La Scola, D. Raoult, and C. Martin.** 2003. Serological hint suggesting that *Parachlamydiaceae* are agents of pneumonia in polytraumatized intensive care patients. Ann. N. Y. Acad. Sci. **990:**311–319.
49. **Greub, G., J. L. Mege, and D. Raoult.** 2003. *Parachlamydia acanthamoebae* enters and multiplies within human macrophages and induces their apoptosis. Infect. Immun. **71:**5979–5985.
50. **Greub, G., J.-L. Mege, J. P. Gorvel, D. Raoult, and S. Méresse.** 2005. Intracellular trafficking of *Parachlamydia acanthamoebae*. Cell Microbiol. **7:**581–589.
51. **Greub, G., P. Berger, L. Papazian, and D. Raoult.** 2003. *Parachlamydiaceae* as rare agents of pneumonia. Emerg. Infect. Dis. **9:**755–756.
51a. **Griffiths, E., A. K. Petrich, and R. S. Gupta.** 2005. Conserved indels in essential proteins that are distinctive characteristics of *Chlamydiales* and provide novel means for their identification. Microbiology **151:**2647–2657.
52. **Groff, J. M., S. E. LaPatra, R. J. Munn, M. L. Anderson, and B. I. Osburn.** 1996. Epitheliocystis infection in cultured white sturgeon (*Acipenser transmontanus*): antigenic and ultrastructural similarities of the causative agent to the chlamydiae. J. Vet. Diagn. Investig. **8:**172–180.
53. **Henning, K., G. Schares, H. Granzow, U. Polster, M. Hartmann, H. Hotzel, K. Sachse, M. Peters, and M. Rauser.** 2002. *Neospora caninum* and *Waddlia chondrophila* strain 2032/99 in a septic stillborn calf. Vet. Microbiol. **85:**285–292.
54. **Hogan, R. J., S. A. Mathews, S. Mukhopadhyay, J. T. Summersgill, and P. Timms.** 2004. Chlamydial persistence: beyond the biphasic paradigm. Infect. Immun. **72:**1843–1855.
55. **Horn, M., A. Collingro, S. Schmitz-Esser, C. L. Beier, U. Purkhold, B. Fartmann, P. Brandt, G. J. Nyakatura, M. Droege, D. Frishman, T. Rattei, H.-W. Mewes, and M. Wagner.** 2004. Illuminating the evolutionary history of chlamydiae. Science **304:**728–730.
56. **Horn, M., and M. Wagner.** 2001. Evidence for additional genus-level diversity of *Chlamydiales* in the environment. FEMS Microbiol. Lett. **204:**71–74.
57. **Horn, M., M. Wagner, K.-D. Müller, E. N. Schmid, T. R. Fritsche, K. H. Schleifer, and R. Michel.** 2000. *Neochlamydia hartmannellae* gen. nov., sp. nov. (*Parachlamydiaceae*), an endoparasite of the amoeba *Hartmannella vermiformis*. Microbiology **146:**1231–1239.
58. **Johnsen, S., N. Birkebaek, P. L. Andersen, C. Emil, J. S. Jensen, and L. Ostergaard.** 2005. Indirect immunofluorescence and real time PCR for detection of *Simkania negevensis* infection in Danish adults with persistent cough and in healthy controls. Scand. J. Infect. Dis. **37:**251–255.
59. **Kahane, S., B. Dvoskin, M. Mathias, and M. G. Friedman.** 2001. Infection of *Acanthamoeba polyphaga* with *Simkania negevensis* and *S. negevensis* survival within amoebal cysts. Appl. Environ. Microbiol. **67:**4789–4795.
60. **Kahane, S., D. Greenberg, M. G. Friedman, H. Haikin, and R. Dagan.** 1998. High prevalence of "Simkania Z," a novel *Chlamydia*-like bacterium, in infants with acute bronchiolitis. J. Infect. Dis. **177:**1425–1429. (Erratum, **178:**1553.)
61. **Kahane, S., N. Platzner, B. Dvoskin, A. Itzhaki, and M. G. Friedman.** 2004. Evidence for the presence of *Simkania negevensis* in drinking water and in reclaimed wastewater in Israel. Appl. Environ. Microbiol. **70:**3346–3351.
62. **Kahane, S., R. Gonen, C. Sayada, J. Elion, and M. G. Friedman.** 1993. Description and partial characterization of a new chlamydia-like microorganism. FEMS Microbiol. Lett. **109:**329–334.
63. **Kocan, K. M., T. B. Crawford, P. M. Dilbeck, J. F. Evermann, and T. C. McGuire.** 1990. Development of a rickettsia isolated from an aborted bovine fetus. J. Bacteriol. **172:**5949–5955.
64. **Kostanjsek, R., J. Strus, D. Drobne, and G. Avgustin.** 2004. '*Candidatus* Rhabdochlamydia porcellionis,' gen. nov., sp. nov., an intracellular bacterium from hepatopancreas of the terrestrial isopod *Porcellio scaber*. Int. J. Syst. Evol. Microbiol. **54:**543–549.
65. **Lewis, D. M., J. Dutkiewicz, W. G. Sorenson, M. Mamolen, and J. E. Hall.** 1990. Microbiological and serological studies of an outbreak of 'humidifier fever' in a print shop. Biodeterioration Res. **3:**467–477.
66. **Lieberman, D., B. Dvoskin, D. V. Lieberman, S. Kahane, and M. G. Friedman.** 2002. Serological evidence of acute infection with the chlamydia-like microorganism *Simkania negevensis* (Z) in acute exacerbation of chronic obstructive pulmonary disease. Eur. J. Clin. Microbiol. Infect. Dis. **21:**307–309.
67. **Lieberman, D., S. Kahane, D. Lieberman, and M. G. Friedman.** 1997. Pneumonia with serological evidence of acute infection with the *Chlamydia*-like microorganism "Z." Am. J. Respir. Crit. Care Med. **156:**578–582.
68. **Longbottom, D., and L. J. Coulter.** 2003. Animal chlamydioses and zoonotic implications. J. Comp. Pathol. **218:**217–244.
69. **Maraha, B., H. Berg, M. Kerver, S. Kranendonk, J. Hamming, J. Kluytmans, M. Peeters, and A. van der Zee.** 2004. Is the perceived association between *Chlamydia pneumoniae* and vascular diseases biased by methodology? J. Clin. Microbiol. **42:**3937–3941.
70. **Marciano-Cabral, F., and G. Cabral.** 2003. *Acanthamoeba* spp. as agents of disease in humans. Clin. Microbiol. Rev. **16:**273–307.
71. **Marrie, T. J., D. Raoult, B. La Scola, R. J. Birtles, E. de Carolis, and Canadian CAP Study Group.** 2001. *Legionella*-like and other amoebal pathogens as agents of community-acquired pneumonia. Emerg. Infect. Dis. **7:**1026–1029.
72. **Maurin, M., A. Bryskier, and D. Raoult.** 2002. Antibiotic susceptibilities of *Parachlamydia acanthamoebae* in amoebae. Antimicrob. Agents Chemother. **46:**3065–3067.
73. **Meijer, A., and J. M. Ossewaarde.** 2002. Description of a wider diversity within the order *Chlamydiales* than currently classified. International Chlamydia Conference, Antalya, Turkey, 16–21 June 2002. [Also available online at http://www.chlamydiae.com.]
74. **Michel, R., B. Haurôder-Philippczyk, K.-D. Müller, and I. Weishaar.** 1992. Observations on Acanthamoebae from nasal mucosa infected by obligate intracellular parasites. Zbl. Bakt. Hyg. Abstr. **325:**56.
75. **Michel, R., K. D. Müller, L. Zöller, J. Walochnick, M. Hartmann, and E. N. Schmid.** 2005. Free-living amoebae serve as host for the chlamydia-like bacterium *Simkania negevensis*. Acta Protozool. **44:**113–121.
76. **Michel, R., K.-D. Müller, and R. Hoffmann.** 2001. Enlarged *Chlamydia*-like organisms as spontaneous infection of *Acanthamoeba castellanii*. Parasitol. Res. **87:**248–251.
77. **Michel, R., K.-D. Müller, E. N. Schmid, and I. Weishaar.** 1994. Acanthamoebae isolated from human nasal mucosa harboured cocci of a new type as obligate intracellular parasites. Trop. Med. Parasitol. **45**(Suppl. II)**:**190.
78. **Michel, R., M. Steinert, L. Zöller, B. Hauröder, and K. Henning.** 2004. Free-living amoebae may serve as hosts for the *Chlamydia*-like bacterium *Waddlia chondrophila* isolated from an aborted bovine foetus. Acta Protozool. **43:**37–42.
79. **Moulder, J. W.** 1991. Interaction of chlamydiae and host cells in vitro. Microbiol. Rev. **55:**143–190.
80. **Ossewaarde, J. M., and A. Meijer.** 1999. Molecular evidence for the existence of additional members of the order *Chlamydiales*. Microbiology **145:**411–417.
81. **Petrich, A., M. Smieja, K. Luinstra, T. Sinha, S. Chong, R. Leigh, M. Loeb, and J. Mahony.** 2002. Development of a PCR assay to determine prevalence of *Simkania negevensis* DNA in specimens from patients with respiratory diseases, p. 471–474. *In* J. Schacter et al. (ed.), Chlamydial infections. Proceedings of the Tenth International Symposium on Human Chlamydial Infections. International Chlamydia Symposium, San Francisco, Calif.
82. **Poppert, S., A. Essig, R. Marre, M. Wagner, and M. Horn.** 2002. Detection and differentiation of chlamydiae by fluorescence in situ hybridization. Appl. Environ. Microbiol. **68:**4081–4089.
83. **Pospischil, A., R. Thoma, M. Hilbe, P. Grest, and J.-O. Gebbers.** 2002. Abortion in woman caused by caprine *Chlamydophila abortus* (*Chlamydia psittaci* serovar 1). Swiss Med. Wkly. **132:**64–66.
84. **Renault, T., and N. Cochennec.** 1995. Chlamydia-like organisms in ctenidia and mantle cells of the Japanese oyster *Crassostrea gigas* from the French Atlantic coast. Dis. Aquat. Organ. **23:**153–159.
85. **Rurangirwa, F. R., P. M. Dilbeck, T. B. Crawford, T. C. McGuire, and T. F. McElwain.** 1999. Analysis of the 16S rRNA gene of microorganism WSU 86–1044 from an aborted bovine foetus reveals that it is a member of the order *Chlamydiales*: proposal of *Waddliaceae* fam. nov., *Waddlia chondrophila* gen. nov., sp. nov. Int. J. Syst. Bacteriol. **49:**577–581.
86. **Schiller, I., A. Schifferli, P. Gysling, and A. Pospischil.** 2004. Growth characteristics of porcine chlamydial strains in different cell culture systems and comparison with ovine and avian chlamydial strains. Vet. J. **168:**74–80.
87. **Schuster, F. L.** 2002. Cultivation of pathogenic and opportunistic free-living amoebas. Clin. Microbiol. Rev. **15:**342–354.

88. **Senn, L., M. Hammerschlag, and G. Greub.** 2005. Therapeutic approaches to *Chlamydia* infection. Expert Opin. Pharmacother. **6:**2281–2290.
89. **Skriwan, C., M. Fajardo, S. Hageler, M. Horn, M. Wagner, R. Michel, G. Krohne, M. Schleicher, J. Hacker, and M. Steinert.** 2002. Various bacterial pathogens and symbionts infect the amoeba *Dictyostelium discoideum*. Int. J. Med. Microbiol. **291:**615–624.
90. **Soldati, G., Z. H. Lu, L. Vaughan, A. Polkinghorne, D. R. Zimmermann, J. B. Huder, and A. Pospischil.** 2004. Detection of mycobacteria and chlamydiae in granulomatous inflammation of reptiles: a retrospective study. Vet. Pathol. **41:**388–397.
91. **Stratton, C. W., and S. Sriram.** 2003. Association of *Chlamydia pneumoniae* with central nervous system disease. Microb. Infect. **5:**1249–1253.
92. **Thao, M. L., L. Baumann, J. M. Hess, B. W. Falk, J. C. K. Ng, P. J. Gullan, and P. Baumann.** 2003. Phylogenetic evidence for two new insect-associated chlamydia of the family *Simkaniaceae*. Curr. Microbiol. **47:**46–50.
93. **von Bomhard, W., A. M. Polkinghorne, Z. H. Lu, L. Vaughan, A. Vogtlin, D. R. Zimmermann, B. Spiess, and A. Pospischil.** 2003. Detection of novel chlamydiae in feline ocular disease. Am. J. Vet. Res. **64:**1421–1428.
94. **Yamaguchi, T., T. Yamazaki, M. Inoue, C. Mashida, K. Kawagoe, M. Ogawa, S. Shiga, Y. Nakagawa, T. Kishimoto, I. Kurane, K. Ouchi, and T. Ohzeki.** 2005. Prevalence of antibodies against *Simkania negevensis* in a healthy Japanese population determined by the microimmunofluorescence test. FEMS Immunol. Med. Microbiol. **43:**21–27.
95. **Yamaguchi, T., T. Yamazaki, M. Inoue, M. Ogawa, S. Shiga, Y. Nakagawa, T. Kishimoto, K. Ouchi,, and T. Ohzeki.** 2004. Factors improving the propagation of *Simkania negevensis* strain Z in cell culture. Jpn. J. Infect. Dis. **57:**103–106.