Protochlamydia naegleriophila as Etiologic Agent of Pneumonia

Nicola Casson,* Rolf Michel,† Karl-Dieter Müller,‡ John David Aubert,* and Gilbert Greub*

Using ameba coculture, we grew a *Naegleria* endosymbiont. Phenotypic, genetic, and phylogenetic analyses supported its affiliation as *Protochlamydia naegleriophila* sp. nov. We then developed a specific diagnostic PCR for *Protochlamydia* spp. When applied to bronchoalveolar lavages, results of this PCR were positive for 1 patient with pneumonia. Further studies are needed to assess the role of *Protochlamydia* spp. in pneumonia.

Recently, a *Naegleria* endosymbiont (KNic) was observed but remained uncultivable, precluding precise identification (*1*). We grew a large amount of strain KNic by using *Acanthamoeba castellanii,* which enabled phenotypic, genetic and phylogenetic analyses that supported its affiliation as *Protochlamydia naegleriophila*. This new ameba-resistant intracellular bacteria might represent a new etiologic agent of pneumonia because it is likely also resistant to human alveolar macrophages (*2*,*3*). Because other *Parachlamydiaceae* were associated with lung infection (*4*–*6*), we assessed the role of *Pr. naegleriophila* in pneumonia by developing a diagnostic PCR and applying it to bronchoalveolar lavages.

The Study

KNic growth in *A. castellanii* was assessed by immunofluorescence (*7*) with in-house mouse anti-KNic and Alexa488-coupled anti-immunoglobulin antibodies (Invitrogen, Eugene, OR, USA). Confocal microscopy (LSM510; Zeiss, Feldbach, Switzerland) confirmed the intracellular location of KNic and demonstrated its rapid growth within *A. castellanii*. To precisely assess the growth rate, we performed PCR on *A. castellanii*/KNic coculture by using PrF/PrR primers and PrS probe. After 60 hours, we observed an increased number of bacteria per microliter of 4 logarithms (online Appendix Figure, available from www.cdc.gov/EID/content/14/1/167-appG.htm).

A. castellanii/KNic and *N. lovaniensis*/KNic cocultures were processed for electron microscopy as described (*7*). Ameba filled with bacteria exhibiting 3 developmental stages already described in other *Parachlamydiaceae* (*8*) were observed (Figure 1).

To measure the serologic differentiation index (SDI) between strain KNic and other *Chlamydia*-like organisms, we immunized Balb/c mice to produce anti-KNic antibodies. Purified *Pr. amoebophila* (ATCC PRA-7), *Simkania negevensis* (ATCC VR-1471), *Parachlamydia acanthamoebae* strain Seine, *Waddlia chondrophila* (ATCC 1470), *Neochlamydia hartmannellae* (ATCC 50802), *Criblamydia sequanensis* (CRIB 18), and *Rhabdochlamydia crassificans* (CRIB 01) antigens were tested by micro-immunofluorescence against mouse anti-KNic antibodies, whereas KNic antigen was tested with serum against all these different *Chlamydia*-like organisms (*9*). SDIs were calculated as described (*9*,*10*). Serum from mice immunized with KNic showed strong reactivity against autologous antigen (titers of 4,096). Significant cross-reactivity between KNic and *Pr. amoebophila* (SDI = 7) and *P. acanthamoebae* (SDI = 10) was observed. Mouse anti-KNic serum did not react with other *Chlamydia*-like organisms (Table 1). Because cross-reactivity between members of the order *Chlamydiales* was proportional to the relatedness between each species (*9*), the strong cross-reactivity between KNic and *Pr. amoebophila* supports the affiliation of KNic in the genus *Protochlamydia*.

Taxonomic position of KNic was further defined by sequencing 16Sr RNA (*rrs*, DQ635609) and ADP/ATP translocase (*nnt*, EU056171) encoding genes. The *rrs* was amplified/sequenced using 16SIGF/RP_2Chlam primers (*11*). The *nnt* was amplified/sequenced using nntF2p (5′-TGT(AT)GAT(CG)CATGGCAA(AG)TTTC-3′) and nntR1p (5′-GATTT(AG)CTCAT(AG)AT(AG)TTTTG-3′) primers. Genetic and phylogenetic analyses were conducted by using MEGA software (*12*). The 1,467-bp *rrs* sequence showed 97.6% similarity with *Pr. amoebophila*, 91.8%–93.2% with other *Parachlamydiaceae*, and 85.7%–88.6% with other *Chlamydiales*. Based on the Everett genetic criteria (*13*), KNic corresponds to a new species within the *Protochlamydia* genus because its sequence similarity with *Pr. amoebophila* is >95% (same genus) and <98.5% (different species). Phylogenetic analyses of *rrs* gene sequences showed that KNic clustered with *Pr. amoebophila*, with bootstraps of 98% and 95% in neighbor-joining and minimum-evolution trees, respectively. The 569-bp *nnt* sequence exhibited 91.1% similarity with *Pr. amoebophila*, 65.5%–72.6% with other *Parachlamydiaceae*, and 55.4%–72.6% with other *Chlamydiales*. Phylogenetic analyses of *nnt* sequences showed that KNic clustered with *Pr. amoebophila*. On the basis of these analyses, we propose to name strain KNic "*Protochlamydia naegleriophila*."

We then developed a specific diagnostic PCR for *Protochlamydia* spp. Primers PrF (5′-CGGTAATACG

*University Hospital Center and University of Lausanne, Lausanne, Switzerland; †Central Institute of the Federal Armed Forces Medical Services, Koblenz, Germany; and ‡Institut für Medizinische Mikrobiologie der Universität Essen, Essen, Germany

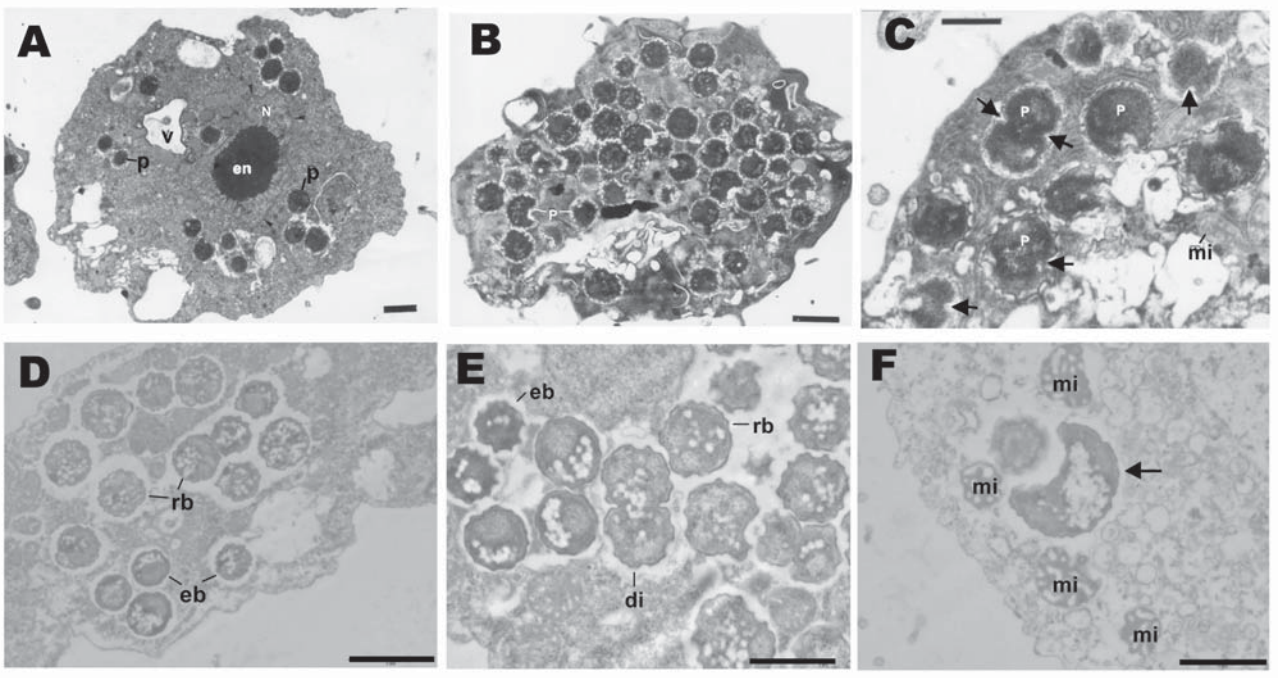

Figure 1. Transmission electron microscopy of *Protochlamydia naegleriophila*. A) *Naegleria lovaniensis* trophozoite after transfer of endocytobionts; strain KNic (p) from the original host strain showing 15 coccoid bacteria distributed randomly within the cytoplasm of the host ameba. N, nucleus; en, endosome (karyosome) within the nucleus; v, empty food vacuoles. Magnification ×10,500; bar = 1 μm. B) *N. lovaniensis* trophozoite jammed with numerous endoparasitic stages of *Pr. naegleriophila*. Magnification ×16,800; bar = 1 μm. C) Enlarged detail of *N. lovaniensis* trophozoite with intracytoplasmic stages of *Pr. naegleriophila*. Some stages show binary fission indicated by the fission furrow (arrows). The endoparasites have a wrinkled gram-negative outer membrane rendering a spiny appearance to the endoparasites. Signs of damage are obvious within the cytoplasm of the host ameba. mi, mitochondria. Magnification ×43,500; bar = 0.5 μm. D) *Pr. naegleriophila* within vacuoles of *Acanthamoebae castellanii* ameba 2 days postinfection. Elementary bodies (eb) and reticulate bodies (rb) are visible. Elementary bodies harbor a smooth membrane compared with the reticulate bodies, which have a spiny shape. Magnification ×10,000; bar = 2 μm. E) Enlarged detail of *A. castellanii* trophozoite with intracytoplasmic stages of *Pr. naegleriophila* 3 days postinfection. Binary fission is observed (di). Magnification ×20,000; bar = 1 μm. F) Crescent body (arrow) within *A. castellanii* observed 3 days postinfection. Magnification ×20,000; bar = 1 μm.

GAGGGTGCAAG-3′) and PrR (5′-TGTTCCGAGGTT GAGCCTC-3′) as well as probe PrS (5′-TCTGACTGAC ACCCCCGCCTACG-3′) were selected. The 5′-Yakima-Yellow probe (Eurogentec, Seraing, Belgium) contained locked nucleic acids (underlined in sequence above). The reactions were performed with 0.2 μM each primer, 0.1 μM probe, and iTaqSupermix (Bio-Rad, Rheinach, Switzerland). Cycling conditions were as described (*14*), and PCR products were detected with ABIPrism7000 (Applied Biosystems, Rotkreuz, Switzerland). Each sample was amplified in duplicate. Inhibition, negative PCR mixture, and extraction controls were systematically tested.

To allow quantification, a plasmid containing the target gene was constructed by cloning PCR products into pCR2.1-TOPO vector (Invitrogen, Basel, Switzerland). Recombinant plasmid DNA quantified using Nanodrop ND-1000 (Witech, Littau, Switzerland) was 10-fold diluted and used as positive controls.

The analytical sensitivity was 10 copy/μL (Figure 2, panel A). Intra-run variability was good (Figure 2B) with a Bland-Altman bias of 0.99 and a limit of agreement of 2.87 (Figure 2, panel A). Inter-run variability was low at high concentration, 1.12, 1.71, 0.82, 1.77 cycles for 10^5, 10^4, 10^3, 10^2 copies/μL, respectively. Inter-run variability was higher at low concentration, 4.22 cycles for 10^1 copies/μL (Figure 2, panel A). Analytical specificity was tested with bacterial and eukaryotic DNA (Table 2). The PCR slightly amplified DNA from *R. crassificans*, another *Chlamydia*-like organism. No cross-amplification was observed with any other bacteria or with human cells. The absence of cross-amplification of *P. acanthamoebae* is important because this *Chlamydia*-related bacteria is considered an emerging agent of pneumonia (*4*–*6*).

We tested 134 bronchoalveolar lavage samples from patients with (n = 65) and without (n = 69) pneumonia and extracted DNA by using a Bio-Rad Tissue Kit. One sample was positive, with 543 and 480 copies/μL. This positive result was confirmed using the 16sigF/16sigR PCR (*13*), which targets another DNA segment. This sequence exhibited 99.6% (284/285) similarity with *Pr. naegleriophila*

Table 1. Antibody titers and serologic differentiation index (SDI) obtained from reciprocal cross-reactions of mouse antiserum with different *Chlamydia*-like organisms, as determined by immunofluorescence*

Strains	Antigen titers (SDI)							
	Pr. naegleriophila	*Pr. amoebophila*	*Simkania negevensis*	*P.a.* strain Seine	*Waddlia chondrophila*	*Neoclamydia hartmannellae*	*Criblamydia sequanensis*	*R. crassificans*
Pr. naegleriophila	4,096 (0)	256 (7)	<4 (14)	512 (10)	4 (20)	16 (14)	<4 (20)	<4 (13)
Pr. amoebophila	32 (7)	256 (0)	<32 (5)	1,024 (3)	64 (10)	32 (0)	128 (9)	64 (3)
S. negevensis	32 (14)	128 (5)	512 (0)	64 (12)	128 (12)	<32 (12)	512 (8)	256 (3)
P. strain Seine	128 (10)	512 (3)	32 (12)	16,384 (0)	64 (19)	64 (12)	<32 (19)	<32 (12)
W. chondrophila	32 (20)	128 (10)	32 (12)	<32 (19)	32,768 (0)	<32 (18)	512 (13)	128 (10)
N. hartmannellae	32 (14)	128 (0)	<32 (12)	128 (12)	<32 (18)	2,048 (0)	<32 (18)	<32 (11)
C. sequanensis	32 (20)	128 (9)	128 (8)	64 (19)	256 (13)	<32 (18)	32,768 (0)	128 (8)
R. crassificans	32 (13)	128 (3)	64 (3)	64 (12)	64 (10)	<32 (11)	256 (8)	256 (0)

**Pr., Protochlamydia*; *P.a., Parachlamydia acanthamoebae*; *R., Rhabdochlamydia.* Titers highlighted in gray, previously published in (*9*), are provided here because these data were used to calculate SDI between *Pr. naegleriophila* strain KNIC and the other *Chlamydia*-related bacteria.

strain KNic and 95.1% (269/283) with *Pr. amoebophila*. The presence of *Protochlamydia* antigen in the sample was confirmed by immunofluorescence performed using rabbit anti-KNic antibody directly on the bronchoalveolar lavage sample and by ameba coculture (online Appendix Figure).

The positive sample was taken from an immunocompromised patient who had cough, dyspnea, and a lung infiltrate. Bronchoscopy examination of the lower respiratory tract showed mucosal inflammation localized at the middle lung lobe. Cytology and Gram stain of the bronchoalveolar lavage showed many leucocytes with macrophages (65%) and neutrophils (23%). Although no antimicrobial treatment was administered prior to bronchoscopy, no other etiologic agent was identified despite extensive microbiologic investigations of bronchial aspirate and bronchoalveolar lavage. Results of Gram stain, auramine stain (for *Mycobacterium* spp.), and silver stain (for *Pneumocystis carinii*) tests were negative. Only physiologic oropharyngeal flora could be grown on sheep-blood and chocolate-bacitracin agars. Cell culture, as well as culture for fungi and mycobacteria, remained sterile. Moreover, results of PCRs specific for the detection of *Legionella pneumophila*, *Chlamydophila pneumoniae*, and *Mycoplasma pneumoniae* (*15*) were all negative. The patient recovered and remained free of symptoms of acute lung infection during the next 20 months.

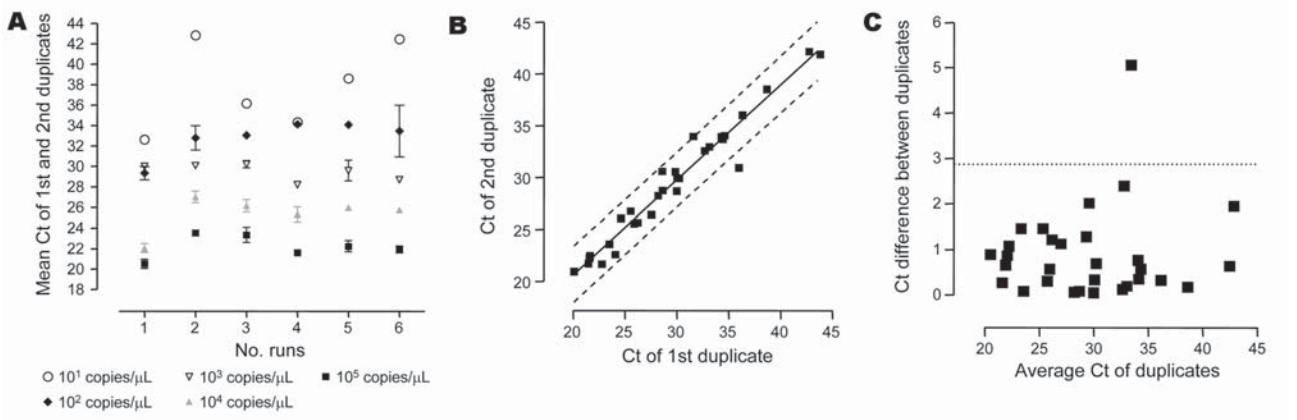

Figure 2. A) Intra and inter-run reproducibility of the real-time PCR assessed on duplicate of plasmidic positive controls performed at 10-fold dilutions from 10^5 to 10^1 plasmid/µL during 6 successive runs. Standard deviations show the intra-run reproducibility of the real-time PCR. B) Plots of the cycle threshold (Ct) of first and second duplicates, showing intra-run and inter-run variability of the real-time PCR between duplicates of positive control. 95% confidence interval is shown by the dashed lines. C) Bland-Altman graph showing the difference of Ct of both duplicates according to the mean of the Ct of duplicates. The dashed line shows the 95% confidence interval (i.e., limit of agreement).

Table 2. Bacterial and eukaryotic DNA used to determine the specificity of the real-time PCR

Bacterial DNA	Source/strain
Bordetella pertussis	Clinical specimen
Chlamydia trachomatis	Clinical specimen
Chlamydophila pneumoniae	ATCC VR-1310
Criblamydia sequanensis	CRIB-18
Enterococcus faecalis	ATCC 29212
Escherichia coli	ATCC 35218
Gardnerella vaginalis	Clinical specimen
Haemophilus influenzae	ATCC 49247
Klebsiella pneumoniae	ATCC 27736
Lactobacillus spp.	Clinical specimen
Legionella pneumophila	Clinical specimen
Listeria monocytogenes	Clinical specimen
Moraxella catharralis	Clinical specimen
Mycobacterium tuberculosis	Clinical specimen
Neisseria lactamica	Clinical specimen
Neisseria weaveri	Clinical specimen
Neochlamydia hartmanella	ATCC 50802
Parachlamydia acanthamoebae strain BN9	ATCC VR-1476
Parachlamydia acanthamoebae strain Hall's coccus	ATCC VR-1476
Protochlamydia amoebophila strain UWE25	ATCC PRA-7
Pseudomonas aeruginosa	ATCC 27853
Rhabdochlamydia crassificans	CRIB-01
Simkania negevensis	ATCC VR-1471
Staphylococcus epidermidis	Clinical specimen
Streptococcus agalactiae	ATCC 13813
Streptococcus mutans	Clinical specimen
Streptococcus pneumoniae	Clinical specimen
Streptococcus pyogenes	ATCC 19615
Waddlia chondrophila	ATCC VR-1470
Eukaryotic DNA	Source/strain
Acanthamoeba castellanii	ATCC 30010
Candida albicans	ATCC 10231
Human cells	ATCC CCL-185

Conclusions

Isolating new species from environmental and clinical samples is important to better define their epidemiology and potential pathogenicity. We defined the taxonomic position of a novel *Naegleria* endosymbiont and proposed its affiliation within the *Protochlamydia* genus as *Pr. naegleriophila* sp. nov. Moreover, we developed a new PCR targeting *Protochlamydia* spp., applied it to clinical samples, and identified a possible role of *Pr. naegleriophila* as an agent of pneumonia.

Protochlamydia naegleriophila (nae.gle.rio′.phi.la Gr. fem.n. *Naegleria*, name of host cell, Gr. adj. *philos, -a* friendly to, referring to intracellular growth of *Protochlamydia naegleriophila* strain KNic within *Naegleria* amebae). The 16Sr RNA sequence (DQ635609) of KNic is 97.6% similar to that of *P. amoebophila,* making this organism a member of the genus *Protochlamydia*. KNic does not grow on axenic media (*1*) but grows by 4 logarithms in 60 h within *A. castellanii*. KNic exhibits a *Chlamydia*-like developmental cycle, with reticulate, elementary, and crescent bodies. The reticulate body is about 900 nm and has a spiny appearance similar to that of *P. amoebophila* (Figure 2, panel B). To be classified within the *Pr. naegleriophila* species, a new strain should show a 16Sr RNA similarity ≥98.5% (*13*) and similar phenotypic traits.

Acknowledgments

We thank J.L. Barblan and the staff of Pôle Facultaire Médical Universitaire at the Medical Faculty of Geneva for assisting with electron microscopy analysis, staff of the Cellular Imaging Facility for assisting with confocal microscopy analysis, Gerhild Gmeiner for technical assistance in preparing *Naegleria lovaniensis* with *Protochlamydia* strain KNic for electron microscopy, Philip Tarr for reviewing the manuscript, and M. Perrenoud and S. Aeby for technical help.

This work was supported by the Swiss National Science Foundation grants FN 3200BO-105885 and FN 3200BO-116445. G.G. is supported by the Leenards Foundation through a career award entitled "Bourse Leenards pour la relève académique en médecine clinique à Lausanne.

Ms Casson is completing a PhD thesis at the University of Lausanne. Her research is dedicated to defining the role of the obligate intracellular *Chlamydia*-like organisms to humans, discovering new species, and defining their role in pneumonia.

References

1. Michel R, Muller KD, Hauröder B, Zöller L. A coccoid bacterial parasite of *Naegleria* sp. (Schizopyrenida: Vahlkampfiidae) inhibits cyst formation of its host but not transformation to the flagellate stage. Acta Protozool. 2000;39:199–207.
2. Greub G, Mege JL, Raoult D. *Parachlamydia acanthamebae* enters and multiplies within human macrophages and induces their apoptosis. Infect Immun. 2003;71:5979–85.
3. Greub G, Raoult D. Microorganisms resistant to free-living amoebae. Clin Microbiol Rev. 2004;17:413–33.
4. Greub G, Raoult D. *Parachlamydiaceae*: potential emerging pathogens. Emerg Infect Dis. 2002;8:625–30.
5. Greub G, Berger P, Papazian L, Raoult D. *Parachlamydiaceae* as rare agents of pneumonia. Emerg Infect Dis. 2003;9:755–6.
6. Greub G, Boyadjiev I, La Scola B, Raoult D, Martin C. Serological hint suggesting that *Parachlamydiaceae* are agents of pneumonia in polytraumatized intensive care patients. Ann N Y Acad Sci. 2003;990:311–9.
7. Casson N, Medico N, Bille J, Greub G. *Parachlamydia acanthamoebae* enters and multiplies within pneumocytes and lung fibroblasts. Microbes Infect. 2006;8:1294–300.
8. Greub G, Raoult D. Crescent bodies of *Parachlamydia acanthamoebae* and its life cycle within *Acanthamoeba polyphaga*: an electron micrograph study. Appl Environ Microbiol. 2002;68:3076–84.
9. Casson N, Entenza JM, Greub G. Serological cross-reactivity between different *Chlamydia*-like organisms. J Clin Microbiol. 2007;45:234–6.
10. Fang R, Raoult D. Antigenic classification of *Rickettsia felis* by using monoclonal and polyclonal antibodies. Clin Diagn Lab Immunol. 2003;10:221–8.

11. Thomas V, Casson N, Greub G. *Criblamydia sequanensis*, a new intracellular *Chlamydiales* isolated from Seine river water using amoeba coculture. Environ Microbiol. 2006;8:2125–35.
12. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 2004;5:150–63.
13. Everett KD, Bush RM, Andersen AA. 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. 1999;49:415–40.
14. Jaton K, Bille J, Greub G. A novel real-time PCR to detect *Chlamydia trachomatis* in first-void urine or genital swabs. J Med Microbiol. 2006;55:1667–74.
15. Welti M, Jaton K, Altwegg M, Sahli R, Wenger A, Bille J. Development of a multiplex real-time quantitative PCR assay to detect *Chlamydia* pneumoniae, *Legionella pneumophila* and *Mycoplasma pneumoniae* in respiratory tract secretions. Diagn Microbiol Infect Dis. 2003;45:85–95.

Address for correspondence: Gilbert Greub, Center for Research on Intracellular Bacteria, Institute of Microbiology, University Hospital Center and University of Lausanne, 1011 Lausanne, Switzerland; email: gilbert.greub@chuv.ch