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New Diagnostic Real-Time PCR for Specific Detection of *Parachlamydia acanthamoebae* DNA in Clinical Samples▽

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Given the low sensitivity of amoebal coculture, we developed a specific real-time PCR for the detection of *Parachlamydia*. The analytical sensitivity was high, and the inter- and intrarun variabilities were low. When the PCR was applied to nasopharyngeal aspirates, it was positive for six patients with bronchiolitis. Future studies should assess the role of *Parachlamydia* in bronchiolitis.

Parachlamydia acanthamoebae is an obligate intracellular bacterium that belongs to the order *Chlamydiales* (1). Epidemiological (2), serological (11, 17), and molecular (6, 7, 10) studies support a potential role of *Parachlamydia acanthamoebae* as an agent of pneumonia. *P. acanthamoebae* has been shown to enter and replicate within human macrophages (13, 14) and to enter and persist within pneumocytes and lung fibroblasts (4). We recently established an animal model of lung infection that confirmed the third and fourth Koch's postulates for the role of *P. acanthamoebae* in pneumonia (3). Taken together, these studies suggest that human exposure to *P. acanthamoebae* may lead to bronchitis, community-acquired pneumonia, and aspiration pneumonia.

Diagnostic methods for the detection of human *Parachlamydia* infection are limited by the inability of these agents to grow on axenic medium. In addition, amoebal coculture is time-consuming and is available in only a few specialized laboratories (12). Serologic diagnosis is also limited by possible cross-reactivity and by the time necessary to seroconvert against an invading pathogen. For these reasons, molecular diagnostic approaches are warranted. Broad-range PCR assays for the members of the *Chlamydiales*, which include *P. acanthamoebae*, have been described (8, 18), but their sensitivities are limited. An additional sequencing step is required, which is directly achievable (without cloning) only for samples containing a minimum of 1,000 DNA copies (G. Greub et al., unpublished data). We therefore developed a real-time PCR assay for the specific detection of *Parachlamydia acanthamoebae* from clinical samples and applied it to samples taken from pediatric patients with bronchiolitis.

Using the primer express software (Applied Biosystems, Darmstadt, Germany), we selected probe PacS (5′-tetrachloro-6-carboxyfluorescein-TTCCACATGTAGCGGTGAAATGCGTAGATATG-Black Hole Quencher 1-3′), as well as primers PacF (5′-CTCAACTCCAGAACAGCATTT-3′) and PacR (5′-CTCAGCGTCAGGAATAAGC-3′), which amplify a 103-bp part of the 16S rRNA-encoding gene. The reactions were performed with 0.2 μM of each primer, 0.1 μM of probe, iTaq Supermix (Bio-Rad, Rheinach, Switzerland), and 5 μl of DNA sample. The cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. The PCR products were detected with an ABI Prism 7000 instrument (Applied Biosystems). Each sample was amplified in duplicate. Inhibition control, negative PCR mixture control, and extraction controls were systematically tested. To allow quantification, a plasmid containing the target gene was constructed, as described previously (5).

The analytical sensitivity of the real-time PCR was 10 copies of plasmidic control DNA per reaction mixture. This sensitivity is similar to that of a quantitative TaqMan PCR targeting the ADP/ATP translocase encoding gene (*tlc*) of *Parachlamydia* (10) (data not shown) and is 100-fold more sensitive than the 16SigF-Rp2Chlam broad-range PCR (18). Use of this real-time PCR has an additional advantage, in that gel electrophoresis is not needed. The risk of amplicon contamination is highly limited since the PCR microplates are not opened after amplification.

The real-time PCR was highly specific, since no cross-amplification was observed when the genomic DNA of humans, fungi (*Candida albicans* ATCC 10231, *Aspergillus fumigatus* clinical isolate), *Acanthamoeba castellanii* (ATCC 30010), and the bacteria listed in Table 1 were tested.

The reproducibility of the threshold cycle (C_T) results was determined by testing duplicates of 10-fold serial dilutions of the plasmid in 11 independent experiments. The intrarun reproducibility was good, as shown in Fig. 1, with the C_T results for both duplicates being relatively similar and with a correlation coefficient (r^2) of 0.961 (Fig. 1A). By using the Bland-Altman test, the 95% confidence interval was 1.32 cycles (Fig. 1B). The interrun reproducibility is shown in Fig. 1C. The interrun variability was relatively low at high concentrations, being 1.43, 1.68, and 1.95 cycles for 10^5, 10^4, and 10^3 plasmidic copies μl^{-1}, respectively. The interrun variability was, however, relatively high at a lower concentration (2.63 cycles for 10^2 plasmidic copies μl^{-1}) (Fig. 1C).

Since several lines of evidence support the role of

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TABLE 1. Bacterial species used to determine specificity of the real-time PCR

Bacterial species	Source or 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 catarrhalis	Clinical specimen
Mycobacterium tuberculosis	Clinical specimen
Neisseria lactamica	Clinical specimen
Neisseria weaveri	Clinical specimen
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

Parachlamydia acanthamoebae as a potential agent of lower respiratory tract infections (reviewed in references 6 and 15), the real-time PCR was applied to 39 nasopharyngeal aspirates obtained from children with respiratory syncytial virus-negative bronchiolitis. DNA was extracted from 200 μl of thawed samples by using the AquaPure genomic DNA extraction kit (Bio-Rad). DNA was eluted in a final volume of 100 μl of the elution buffer provided with the kit. A negative extraction control was tested for each extraction run. The results for positive samples were confirmed by the *tlc* real-time PCR (10).

Parachlamydia DNA was detected in 13 of the 39 samples, 6 of which were confirmed to be positive by the *tlc* quantitative PCR. The clinical and microbiological characteristics of these six patients are summarized in Table 2. We successfully sequenced the product of the 16SigF-Rp2Chlam PCR (18) only once, consistent with a bacterial burden of <1,000 copies in the five other samples. The sequence shared 99.6% (577/579) similarity with *P. acanthamoebae* strain Hall's coccus and 100% (577/577) similarity with *P. acanthamoebae* strain BN9.

The seven patients with a positive result by the PacF-PacR PCR (the new real-time PCR) but a negative result by the real-time PCR targeting the *tlc* gene were also negative by the 16SigF-Rp2Chlam PCR. Thus, the positive PacF-PacR PCR results may represent either false-positive results due to PCR contamination or false-negative results by both of the other PCRs.

The fact that another agent of bronchiolitis was identified in only one patient positive for *Parachlamydia* (Table 2) supports a possible role of *Parachlamydia* in the pathogenesis of bronchiolitis. However, we cannot exclude the possibility that *Parachlamydia* is only a colonizer of the lower respiratory tract. Since *Simkania negevensis*, another member of the order *Chlamydiales* related to *Parachlamydia*, has been associated with bronchiolitis in infants (9, 16), further studies should investigate a possible pathogenic role of *P. acanthamoebae* in this setting. This new quantitative PCR may be useful for the better definition of the pathogenicity of *Parachlamydia* in both animals and humans.

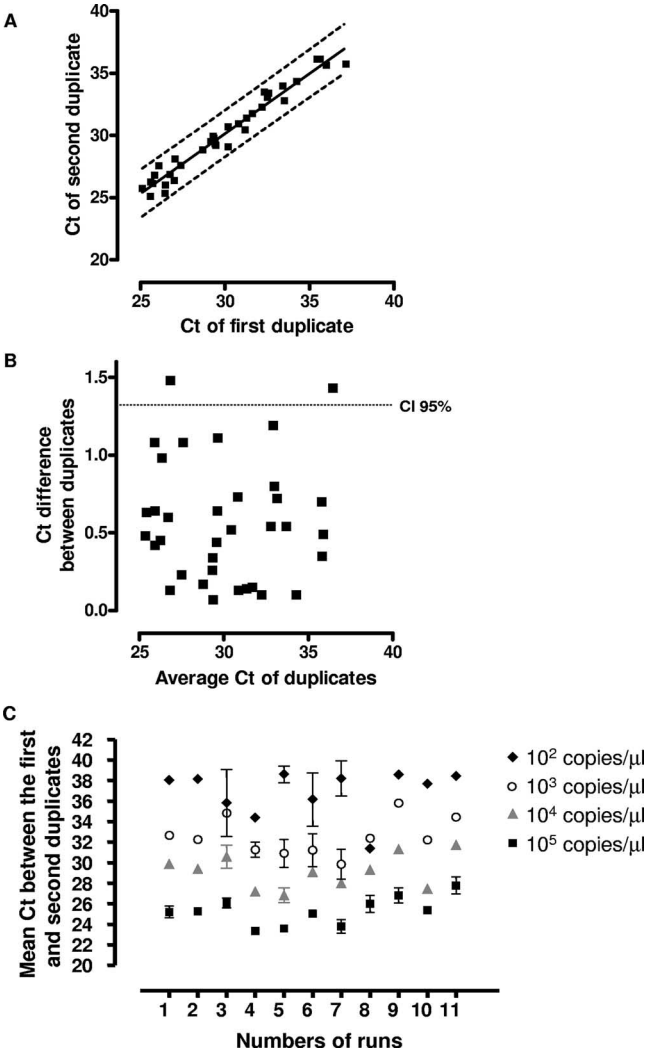

FIG. 1. (A) Plots of the C_T values of the first and second duplicates, showing the intrarun and interrun variabilities of the real-time PCR between duplicates of the positive control. The dashed lines show the 95% confidence interval. (B) Bland-Altman graph showing the difference in the C_T values of both duplicates according to the mean of the C_T values of the duplicates. The dashed line shows the 95% confidence interval (i.e., the limit of agreement). (C) Intra- and interrun reproducibilities of the real-time PCR assessed with duplicates of plasmidic positive controls performed at 10-fold dilutions from 10^5 to 10^2 plasmid μl^{-1} in 11 successive runs. Standard deviations show the intrarun reproducibility of the real-time PCR.

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TABLE 2. Characteristics of six patients with bronchiolitis whose nasopharyngeal samples had positive *Parachlamydia* PCR results[a]

Patient no.	Age	Sex	Signs and symptoms	X-ray findings	Paraclinical findings	Underlying condition(s)	Other etiology	New qPCR result (mean C_T value [no. of copies/μl])	*tlc* qPCR result (mean C_T value)	16SigF-Rp2Chlam PCR result
1	10 mo	M	Increased work of breathing, desaturation, hypercapnia	Lung infiltrate, emphysema	Normal leukocyte count and C-reactive protein level	Surgically corrected congenital heart defect, tracheomalacia	Respiratory syncytial virus detected 1 wk later	33.48 (1,049)	32.49	Positive[b]
2	14 yr	M	Increased work of breathing, desaturation	Diffuse infiltrates				34.79 (641)	38.79	Negative
3	26 mo	F	Increased work of breathing, cough	Peribronchial infiltrates		Asthma		36.28 (220)	36.96	Negative
4	9 mo	M	Increased work of breathing, low-grade fever	Right upper lobe atelectasis	Normal leukocyte count and C-reactive protein level			35.33 (289)	36.10	Negative
5	2.5 yr	M	Increased work of breathing, cough, rhinorrhea	X ray not done				36.75 (157)	38.09	Negative
6	11 mo	F	Increased work of breathing, fever, cough, rhinorrhea	Right lower lobe pneumonia and infiltrate	Increased C-reactive protein level, leucocytosis with left shift	Acute otitis media		37.86 (81)	40.17	Negative

[a] qPCR, quantitative TaqMan real-time PCR; M, male; F, female.
[b] The sequence shared 99.6% (577/579) similarity with *P. acanthamoebae* strain Hall's coccus.

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