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BRIEF REPORT

Development of a real-time PCR for the specific detection of *Waddlia chondrophila* in clinical samples

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Abstract *Waddlia chondrophila* is considered as an emerging human pathogen likely involved in miscarriage and lower respiratory tract infections. Given the low sensitivity of cell culture to recover such an obligate intracellular bacteria, molecular-based diagnostic approaches are warranted. We thus developed a real-time PCR that amplifies *Waddlia chondrophila* DNA. Specific primers and probe were selected to target the 16S rRNA gene. The PCR specifically amplified *W. chondrophila* but did not amplify other related-bacteria such as *Parachlamydia acanthamoebae, Simkania negevensis* and *Chlamydia pneumoniae*. The PCR exhibited a good intra-run and inter-run reproducibility and a sensitivity of less than ten copies of the positive control. This real-time PCR was then applied to 32 nasopharyngeal aspirates taken from children with bronchiolitis not due to respiratory syncytial virus (RSV). Three samples revealed to be *Waddlia* positive, suggesting a possible role of this *Chlamydia*-related bacteria in this setting.

Introduction

During the last 15 years, several *Chlamydia*-related bacteria have been discovered [1–3]. Among them, *Simkania negevensis* and *Parachlamydia acanthamoebae* seem to be implicated in bronchiolitis in children and pneumonia in adults [4, 5]. *Waddlia chondrophila* was isolated twice from aborted bovine fetuses [6, 7] and a seroepidemiological study revealed a strong association between *W. chondrophila* seropositivity and miscarriage in humans [8]. In addition, DNA of *W. chondrophila* was amplified from the sputum of a patient suffering from pneumonia [9]. Moreover, *W. chondrophila* showed rapid replication in human macrophages [10]. Detection of this obligate intracellular bacterium is limited by its inability to grow on axenic media. We thus developed a real-time diagnostic PCR assay to specifically detect *W. chondrophila* DNA and applied it to samples from patients with bronchiolitis.

Material and methods

Using Primer3 software [11], a forward primer WadF4 (5′-GGCCCTTGGGTCGTAAAGTTCT-3′), a reverse primer WadR4 (5′CGGAGTTAGCCGGTGCTTCT-3′) and a probe WadS2 (5′-FAM-CATGGGAACAAGAGAAGGATG-BHQ-3′) were selected to amplify a 101-bp fragment of the 16S rRNA gene of both *W. chondrophila* strains available to date: strain ATCC VR-1470 (LGC Promochem, Molsheim, France) and strain 2032/99 (K. Sachse, Jena, Germany). The probe contained locked nucleic acids (underlined in sequence above).

Both strains were grown in *Acanthamoeba castellanii* ATCC 30010 and purified on sucrose and gastrographin, as previously described [12]. Bacterial DNA was then extracted using QIAamp DNA Mini kit (Qiagen, Hilden, Germany). A 16S rRNA gene plasmid positive control was constructed using primers WadF3 (5′-CAGTCGA GAATCTTTCGCAAT-3′) and WadR4, as described [13].

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Table 1 Bacterial species used to test the specificity of the *Waddlia* real-time PCR

Bacterial species	Source/strain
Bacteroides fragilis	ATCC25825
Chlamydia suis	S45/6 [a]
Chlamydia abortus	S26/3 [b]
Chlamydia pecorum	W73 [a]
Chlamydia psittaci	T49/90 [c]
Chlamydia pneumoniae	K6 [d]
Criblamydia sequanensis	CRIB-18
Escherichia coli	ATCC 25922
Haemophilus influenzae	ATCC 49247
Legionella pneumophila	Clinical specimen [e]
Moraxella catharralis	Clinical specimen [e]
Neochlamydia hartmannellae	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 aureus	ATCC 25923
Streptococcus mitis	ATCC 6249

[a] Kindly provided by J. Storz, Baton Rouge Louisiana, LA, USA

[b] Kindly provided by G.E. Jones, Moredun Research Institute, Edinburgh, UK

[c] Kindly provided by R.K. Hoop, Zürich, Switzerland

[d] Kindly provided by A. Pospischil, Zürich, Switzerland

[e] Kindly provided by Christian Durussel, Lausanne, Switzerland

PCR reactions were performed with 0.2 μM of each primer, 0.1 μM of probe and iTaq supermix with ROX (BioRad, Reinach, Switzerland). Cycling conditions were 2 min at 50°C, 3 min at 95°C followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Amplification and detection of PCR products were performed with ABI Prism 7000 (Applied Biosystems, Foster City, USA). To assess the analytical specificity of this new real-time PCR, we tested DNA extracted from 20 different bacterial strains (Table 1). We also tested DNA of *A. castellanii* ATCC 30010 and human DNA (Roche Diagnostics, Mannheim, Germany). A PCR inhibition control (4 μl DNA spiked with 1 μl positive control) was used for all these DNA to verify that absence of amplification was due to the specificity of the PCR and not to the presence of PCR inhibitors. To assess the analytical sensitivity, 10-fold serial dilutions (10^5 to 10^0 copies) of the positive plasmid control were tested in duplicates in 15 different runs.

Intra-run reproducibility was assessed on 75 duplicates by plotting the Ct (threshold cycle) values of both duplicates against each other on a graph, by plotting the differences between both duplicates against the mean of each duplicate and by calculating the 95% confidence interval using the Bland-Altman algorithm (GraphPad 5.0). To assess the inter-run reproducibility, the mean Ct of duplicates obtained in 15 independent runs were compared for each concentration of plasmid DNA.

Results and discussion

Genomic DNA of the two *Waddlia* strains available in co-culture were successfully amplified by this 16S rRNA gene real-time PCR with Ct values of 19 when samples with approximately 10^5 DNA copies/μl were tested. The analytical sensitivity of the real-time PCR was of about ten copies of plasmid control DNA. Indeed, when testing ten-fold dilutions of plasmid, nine of 15 replicates were positive at a concentration of one copy whereas all 15 replicates were positive at a concentration of ten copies. Although the assay is essentially based on the specificity of the probe, analytical specificity was high. No cross-amplification was observed when testing microorganisms listed in Table 1, which indicates that the specificity of the probe ensured a high degree of discrimination between the amplicons of *Waddlia chondrophila* and those of the other tested bacteria. Intra-run reproducibility was high as shown

Fig. 1 **a** Plot of cycle threshold (Ct) values of first duplicate versus second duplicate showing intra-run reproducibility. The *solid line* represents the linear regression whereas the *dashed lines* represent the 95% prediction interval. **b** Bland-Altman graph representing the Ct difference between both duplicates according to the average Ct of duplicates. The *dashed line* represents the 95% confidence interval (CI 95%). **c** Inter-run and intra-run reproducibilities assessed using 10^1 to 10^5 positive control plasmid copies in 15 successive runs. Error bars represent the standard error of the mean of duplicates (intra-run reproducibility)

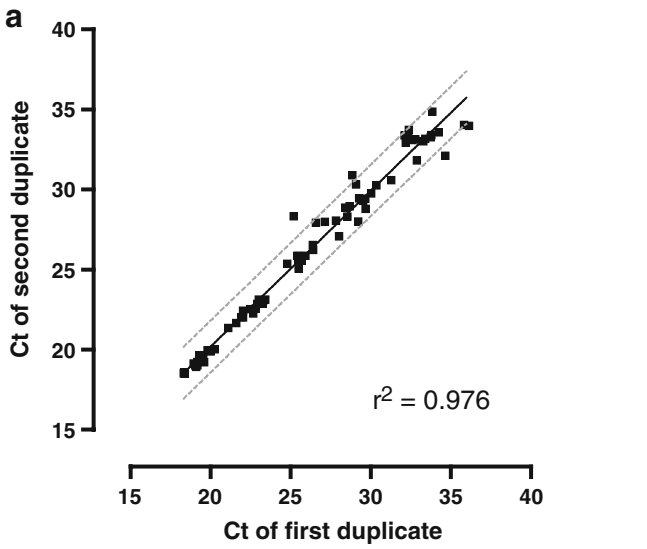

Table 2 Description of three patients with bronchiolitis not due to respiratory syncytial virus, who exhibited a positive *Waddlia* PCR on nasopharyngeal samples

Age	Gender	Signs and symptoms	X-ray findings	Paraclinical findings	Underlying conditions	Other etiology	Real-time PCR results (mean Ct values and DNA copies)
1 month	Female	Vomiting, cough	No X-ray	WBC normal, CRP <10, negative urine culture	-	No	37.9 (8.1 copies)
11.5 years	Female	Fever, cough, headache, fatigue	Sinus normal, No thorax X-ray	WBC 8.4, CRP<10, negative streptotest	Developmental delay, obesity, enuresia	No	38.1 (6.7 copies)
1 year	Female	Fever, cough, vomiting	Interstitial infiltrate partially confluent to the apex and right base compatible with interstitial pneumonia	WBC 7.2, left shift with 39.5% of non segmented neutrophils, CRP=80	Acute otitis media	Influenza A and parainfluenza type 3[a]	33.6 (118.2 copies)

Ct cycle threshold, *WBC* white blood cells, *CRP* C-reactive protein

[a] Negative for adenovirus, influenza B and parainfluenza 1 and 2

in Fig. 1, with Ct of both duplicates being relatively similar with a correlation coefficient r^2 of 0.976 (Fig. 1a). In Fig. 1b, the Bland-Altman graph revealed that the 95% confidence interval was 1.72 cycles. Inter-run reproducibility is shown in Fig. 1c. The standard deviation values reflect small deviation from the mean and indicate good reproducibility among different runs. Thus, the quantification was highly reliable from 10^1 to 10^5 copies, with an average difference of 3 cycles per ten-fold dilution (Fig.1c).

The sensitivity of this *W. chondrophila* real-time PCR, which was less than ten copies, is excellent and comparable to other diagnostic TaqMan PCR assays [14, 15]. This real-time PCR is more sensitive than the 16SigF-Rp2Chlam broad range PCR [16], which only detects about 1,000 copies of DNA [14]. Thus, this real-time PCR allows detection of *Waddlia* DNA not detectable by classical PCR. This increased sensitivity is likely a result of the real-time technology, which combines amplification of small length amplicons and detection with an highly sensitive fluorescent probe.

As the role of *W. chondrophila* in lower respiratory tract infections is possible [9], we applied our real-time PCR to 32 nasopharyngeal aspirates from children with bronchiolitis not due to respiratory syncytial virus (RSV), which is the most common etiology of bronchiolitis. *Waddlia* DNA was detected in three samples. The characteristics of these three patients are summarized in Table 2. Briefly, these three female patients all exhibited symptoms of bronchiolitis. Another microbial etiology was identified in one of the three patients.

In conclusion, we developed a new *W. chondrophila* real-time PCR. The assay revealed to be specific with a very low detection limit and its application to clinical samples allowed detection of *W. chondrophila* DNA in three samples taken from children with bronchiolitis. However, more patients should be investigated in order to confirm a possible role of *W. chondrophila* in bronchiolitis. In the future, our *Waddlia* diagnostic PCR could be useful to define the role of *Waddlia* in other human diseases, such as pneumonia and miscarriage. Since the format of this real-time PCR assay is compatible with 384-well plates, the test will also be useful for screening large numbers of samples to investigate the public health risk of this amoeba-resisting bacterium.

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