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Development of a new *Chlamydiales*-specific real-time PCR and its application to respiratory clinical samples

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Abstract

Originally composed of the single family *Chlamydiaceae*, the *Chlamydiales* order has extended considerably in the last decades. *Chlamydia*-related bacteria were added and classified in 6 different families and family-level lineages: the *Criblamydiaceae, Parachlamydiaceae*, *Piscichlamydiaceae*, *Rabdochlamydiaceae, Simkaniaceae* and *Waddliaceae*. While several members of the *Chlamydiaceae* family are known pathogens, recent studies showed diverse associations of *Chlamydia*-related bacteria with human and animal infections. Some of these latter bacteria are preoccupying since, given their ability to replicate in free-living amoebae, they may also replicate efficiently in other phagocytic cells, including cells of the innate immune system. Thus, a new *Chlamydiales*-specific real-time PCR targeting the conserved 16S rRNA gene was developed. This new molecular tool can detect at least 5 DNA copies and show very high specificity without cross-amplification from other bacterial clade DNA. The new PCR was validated with 128 clinical samples positive or negative for *Chlamydia trachomatis* or *C. pneumoniae*. Among 65 positive samples, 61 (93.8%) were found positive with the new PCR. The 4 discordant samples, re-tested with the original test, were negative or below detection limits. Then, the new PCR was applied to 422 nasopharyngeal swabs taken from children with and without pneumonia: 48 (11.4%) samples were positive, of which 45 were successfully sequenced. The majority of the sequences corresponded to *Chlamydia*-related bacteria and especially to members of the *Parachlamydiaceae* family.

INTRODUCTION

The *Chlamydiales* order contains obligate intracellular bacteria separated in 7 different families and family-level lineages, the *Chlamydiaceae*, the *Criblamydiaceae*, the *Parachlamydiaceae*, the *Piscichlamydiaceae*, the *Rhabdochlamydiaceae*, the *Simkaniaceae* and the *Waddliaceae* (16, 23-25). Some of these bacteria are established pathogens and for instance, *Chlamydia trachomatis, C. psittaci* and *C. pneumoniae* from the *Chlamydiaceae* family can cause significant human infections. The others families constitute a group called *Chlamydia*-related bacteria (also referred as *Chlamydia*-like organisms), which has been yet poorly investigated. Like the *Chlamydiaceae*, these *Chlamydia*-related bacteria are obligate intracellular bacteria that also exhibit a biphasic developmental cycle. Serological and molecular studies have implicated some species in various human and animal infections. *Parachlamydia acanthamoebae* is associated with human pneumonia (6, 12, 26, 27) and might cause bovine abortions (5, 38, 39), *Simkania negevensis* is responsible of respiratory infections, especially in children (18, 20, 22, 28, 32-35) whereas *Waddlia chondrophila* has been reported to cause abortion in bovines (14, 40) and is strongly suspected as an agent of miscarriage in human (3, 4). Some of these newly discovered *Chlamydia*-related bacteria that resist digestion by several environmental amoebae are also resistant to professional phagocytes of the innate immune system such as macrophages. Considering their potential threat on human health, it is important to be able to detect these obligate intracellular bacteria, since classical culture methods are ineffective. Thus, quantitative real-time PCRs have been developed (6, 21, 26, 31, 42), however they target specifically only one single species. Moreover, the only “broad-range” quantitative real-time PCR previously developed

in the field is a family-specific PCR amplifying DNA from members of the *Chlamydiaceae* family, which will not allow detection of *Chlamydia*-related bacteria (17). Since the biodiversity of *Chlamydiales* appears to be much larger than previously expected and new chlamydial strains are constantly discovered (7-9, 29, 30, 41), a molecular diagnostic tool able to detect any member of the *Chlamydiales* order is needed. Such a molecular tool would help in identifying the potential pathogenic role of *Chlamydia*-related bacteria and in specifying the true diversity of *Chlamydiales*, which is likely yet underestimated.

Thus, we developed a *Chlamydiales*-specific real-time Taqman PCR (hereafter named pan-*Chlamydiales* PCR), that we validated using 128 clinical samples available from previous studies. We also applied this new PCR to 422 nasopharyngeal swabs samples taken from children with and without pneumonia, to investigate for the presence of chlamydial DNA.

MATERIAL AND METHODS

DNA extraction. Nasopharyngeal swabs samples were extracted automatically with the LC automated system (Roche, Rotkreuz, Switzerland) and the MagNA Pure LC DNA isolation kit I (Roche). Extracted DNAs were re-suspended in 100 µl of the provided elution buffer. One negative extraction control was included for each extraction run (32 wells/extraction run).

Primers and probe. Based on an alignment of the 16S ribosomal RNA sequences available in Genbank database (http://www.ncbi.nlm.nih.gov/genbank/), specific primers and probe were designed using the Geneious software 5.0.3 and primer3Plus (37). Locked Nucleic Acids (underlined below) were added to ensure a higher specificity. We chose a primer forward panCh16F2 (5'-CCGCCAACACTGGGACT-3'), a primer reverse panCh16R2 (5'-GGAGTTAGCCGGTGCTTCTTTACC-3') and a probe panCh16S (5'-FAM [6-carboxyfluorescein]-

CTACGGGAGGCTGCAGTCGAGAATC-BHQ1 [Black Hole Quencher]-3'), targeting a fragment of about 207 to 215 bp in the 16S ribosomal RNA gene (length variable according to the species).

Real-time PCR assay. PCR assays were performed in 20 µl, with iTaq supermix with ROX (BioRad, Reinach, Switzerland), 0.1 µM of each primer (Eurogentec, Seraing, Belgium), 0.1 µM of probe (Eurogentec), molecular biology grade water (Sigma-Aldrich, Buchs, Switzerland) and 5 µl of DNA sample. Cycling conditions were 3 min at 95°C, followed by 50 3-steps cycles of 15 s at 95°C, 15 s at 67°C and 15 s at 72°C. PCR products, tested in duplicate, were detected with a StepOne instrument (Applied Biosystems, Zug, Switzerland) for children nasopharyngeal swabs and with a ABI 7900 (Applied Biosystems) for analytic validation on samples from the retrospective study. Water was used as a negative PCR control.

Quantification and positive recombinant plasmid control. DNA from *Parachlamydia acanthamoebae* strain Hall's coccus was isolated from a purified bacterial culture available in our laboratory, using the Wizard Genomic DNA purification kit (Promega, Duebendorf, Switzerland). A PCR reaction was performed using the polymerase AmpliTaq Gold (Applied Biosystems) and the primers Pacstd16SF2 (5'-GCTGACGGCGTGGATGAGGC-3') and Pacstd16SR2 (5'-CCTACGCGCCCTTTACGCCC-3'). The PCR products were purified with the MSB Spin PCRapace kit (Invitek, Berlin, Germany) and cloned according to the manufacturer's protocol, in the pCR2.1-TOPO vector (Invitrogen, Basel, Switzerland) containing ampicilin and tetracycline resistance genes. Isolation of plasmidic DNA was performed with the QIAprep Spin Miniprep Kit (Qiagen, Kombrechtikon, Switzerland). The construction was checked by sequencing, using primers of the pCR2.1-TOPO vector

provided in the kit. Quantification of the recombinant plasmid was done on a Nanodrop ND-1000 (Witech, Littau, Switzerland), and serial dilutions (10^5 to 10^0 copies/µl) were used as positive controls, to establish a standard curve for quantification and to check the reproducibility and efficiency of detection (see below). Negative controls, standard curve and samples were all analyzed in duplicate.

Analytical specificity, efficiency and reproducibility of the PCR

The specificity of the new quantitative PCR was tested using DNA extracted from different bacteria commonly found in respiratory tract samples (Table 1). DNAs were diluted at 10^5 copies of the 16*S* rRNA gene per reaction. Using the positive control plasmid, the analytical sensitivity and the reproducibility of the PCR was assessed on duplicates with 10-fold dilutions ($5x10^5$ to $5x10^0$ copies/reaction) in 12 independent runs. The efficiency of detection was performed with the positive control plasmid diluted at 50, 20, 5, 1 and 0.5 DNA copies per reaction; each concentration tested in 20 replicates. The range of the PCR was also evaluated with chlamydial DNA from 15 different strains (Table 2).

Clinical samples. The new pan-*Chlamydiales* PCR was validated on 128 clinical samples. Different clinical samples including urines, cervico-vaginal, anorectal and nasopharyngeal swabs were collected and DNA was extracted between 2004 and 2010 by the diagnostic laboratory of the Institute of Microbiology, Lausanne, Switzerland (Table 3). These samples were originally tested with a real-time PCR specific of *Chlamydia trachomatis* (113 samples) (2, 13) and with a multiplex real-time PCR (42) detecting specifically *Chlamydia pneumoniae* but also *Mycoplasma pneumoniae* and *Legionella pneumophila* (15 samples). Positive samples for *M. pneumoniae* (5 samples) or *L. pneumophila* (3 samples) were included to confirm the high specificity of the new real-time PCR. We then applied our new

pan-*Chlamydiales* PCR to 422 nasopharyngeal swabs prospectively collected between 2008 and 2010 at the University Hospitals of Geneva from children with (n=265) or without (n=157) pneumonia. Pneumonia was defined by the presence of at least one of the following symptoms: fever (>38°C), cough, dyspnea, tachypnea and an infiltrate or a consolidation at the lung X-ray. All samples were systematically tested for the following viruses by PCR: respiratory syncytial virus A, B, adenovirus A, B, C and E, coronavirus HKU1, OC43, 229E, NL63, parainfluenzae virus 1, 2 and 3, HMPV A, B, enterovirus A, B, C, D, rhinovirus A, B, influenza virus A, B and H1N1 for some samples during the 2009 epidemics. In addition, the nasopharyngeal samples were tested by PCR for the presence of *Streptococcus pneumoniae* and by real-time PCR for *Mycoplasma pneumoniae* and *Legionella pneumophila.* Children were aged between 1 and 15 years old: median age in the group of pneumonia was 4.6 years old and 6.2 years old for the control group. All DNA samples were tested in duplicate. Positive samples were systematically confirmed in a second run. To test for potential false negative results due to PCR inhibitors, an inhibition test was systematically performed with 4 µl of clinical DNA samples and 1 µl of the positive control at a concentration of 200 DNA copies/µl. The PCR was considered inhibited when the quantification was below 50 DNA copies per reaction (four-fold reduction). Moreover, a total of 60 non inoculated swabs (Copan, Brescia, Italy) were used as an additional negative control, to check that the commercial swabs used in the prospective study were not contaminated with any chlamydial DNA.

Sequencing of positive samples. Amplicons of positive samples were purified using the MSB Spin PCRapace kit (Invitek). A sequencing PCR was performed with specifically designed inner primers panFseq (5'-CCAACACTGGGACTGAGA-3') and panRseq (5'-

GCCGGTGCTTCTTTAC-3'). The sequencing PCR assay was done using the BigDye® Terminator v 1.1 Cycle seq kit (Applied Biosystems). Sequences of positive nasopharyngeal samples taken from children have been deposited on the NCBI website. Accession numbers are HQ721193 to HQ721240.

RESULTS

Sensitivity and specificity of the pan-*Chlamydiales* quantitative PCR

No cross-reaction was observed with the different bacterial or amoebal strains tested (Table 1). A competition test was also performed by testing an increasing amount of DNA from *Protochlamydia naegleriophila* strain KNic (from 0 to 10^3 copies of the 16S rRNA gene per reaction) in the presence of an increasing amount of a mixture of non chlamydial DNA (Table 1) (from 0 to 10^6 copies of the 16S rRNA gene per reaction). The amplification of the DNA from *P. naegleriophila* strain KNic was not affected by competing non chlamydial DNA up to 10^5 copies of the 16S rRNA gene of non targeted bacteria, demonstrating the high specificity of the PCR. The range of the new PCR was evaluated with 15 DNAs from different chlamydial strains (Table 2). As expected, all the different members of the *Chlamydiales* order tested were detected, confirming the large range of the PCR. Despite the presence of 1 mismatch with the probe in the 16S rDNA sequence of *C. psittaci* and *C. abortus*, both species were successfully amplified. Alignment of all other sequences available from members of the Chlamydiales order demonstrated that 1 mismatch is also present for *C. caviae, C. felis*, and Candidatus *Clavochlamydia salmonicola* in the probe or for *Rhabdochlamydia porcellionis* and *R. crassificans* in the forward primer. These species are nevertheless likely all amplified with our new pan-*Chlamydiales* PCR. Indeed, numerous

DNAs somehow related to *Rhabdochlamydiaceae* have been successfully amplified from clinical samples (see below). The only known member of the *Chlamydiales* order likely not amplified using our pan-*Chlamydiales* PCR is *Piscichlamydia salmonis*, since as many as 6 mismatches are present

Reproducibility and efficiency of the pan-*Chlamydiales* real-time PCR

The inter-run and intra-run reproducibility was assessed respectively on 12 independent runs and 72 duplicates, which results are shown in Figure 1 (A and B). All duplicates were amplified for 50 and more DNA copies per reaction and 18 replicates out of 24 (75%) for 5 DNA copies per reaction. The Bland-Altman graph clearly indicates that differences between duplicates were below 1 cycle threshold (Ct) for DNA copies above 50 per reaction, demonstrating a high reproducibility. The efficiency of detection was evaluated on 20 replicates for 50, 20, 5, 1 and 0.5 DNA copies per reaction. The PCR showed 100% detection for 50 and 20 DNA copies, 75%, 30% and 5% for 5, 1 and 0.5 DNA copies per reaction respectively (Fig. 1C).

Analytical validation of the new PCR

Over the 65 samples positive for *Chlamydia trachomatis* or *C. pneumoniae*, 61 (93.8%) samples were found positive with the new PCR (Table 3A and B). The 4 discordant samples were originally positive for *C. trachomatis*, from anorectal swabs (n=2), urine (n=1) and ascitis liquid (n=1). These 4 samples were tested a second time with the original test (*C. trachomatis* real-time PCR) and were found negative (n=1) or positive with only 0.2, 6 and 1.2 copies per reaction, which was most certainly below the detection limits of the pan-*Chlamydiales* PCR. Seven positive samples with the pan-*Chlamydiales* PCR (cycle threshold values from 23.6 to 41.3) were sequenced to confirm the results. All the sequences obtained

showed 100% similarity with the expected species (Supplementary table S1), confirming the specificity of the new PCR and the possible identification by sequencing even with later cycle threshold values. Positive samples for *Mycoplasma pneumoniae* (n=5) and *Legionella pneumophila* (n=3) were all found negative with the new PCR (Table 3A). On the total of 63 samples negative for *C. trachomatis* or *C. pneumonia*e, only 1 sample was amplified, showing 92% with the closest previously described *Protochlamydia naegleriophila* strain CRIB 41 (FJ532294.1) (Table 3B).

Application of the quantitative PCR

The application of the new pan-*Chlamydiales* PCR was on 422 nasopharyngeal swabs samples taken from children revealed 48 positive samples: 31 (7.3%) samples with 1/4 positive wells, 6 (1.4%) samples with 2/4 positive wells, 4 (0.9%) samples with 3/4 positive wells and 7 (1.7%) samples with 4/4 positive wells (Supplementary Table S2). A correlation between the cycle threshold value (Ct) and the number of positive wells was observed (Supplementary figure S1). Samples with <5 DNA copies per reaction (high Ct values) were amplified in 3/4, 2/4 and 1/4 wells. The 48 positive samples were sequenced and 48 sequences were obtained from 45 different patients (Supplementary Table S2). Indeed, the sequencing of 3 samples failed and for 3 others samples, 2 different sequences were obtained (Patients GE10169, HE210023, HE210045, see Table S2). Thus 94% of the positive samples were successfully sequenced. Patients' characteristics and sequencing results for patients with pneumonia are presented in Table 4. Among these 25 patients listed in Table 4, another etiology was identified for only 8 patients.

A percentage of similarity for the best BLAST greater than 90% was observed for all the 48 sequences obtained, allowing identification at least at the family level. On the 48 sequences

obtained, 26 belonged to the *Parachlamydiaceae* family, 7 to the *Chlamydiaceae* family, 5 to the *Simkaniaceae* family, 5 to the *Criblamydiaceae* family, 3 to the *Rhabdochlamydiaceae* family, 1 seemed to belong to the novel E6-lineage (7, 11) and 1 other sequence corresponded to an unclassified *Chlamydiales* (Table S2). Among the 7 sequences corresponding to a *Chlamydiaceae* species, 6 showed 100% similarity with *Chlamydia pneumoniae:* 5 samples were taken from children with pneumonia (Table 4) whereas 1 was taken from an apparently healthy child (Supplementary Table S2). This latter patient had a previous history of obstructive bronchitis and chronic otitis media. The remaining *Chlamydiaceae* sequence showed 100% similarity with *C. trachomatis* (the sample was taken from a child with pneumonia) (Table 4). Among the 26 sequences corresponding to a member of the *Parachlamydiaceae* family, 10 (40%) were taken from 10 patients with pneumonia and 16 (60%) from 15 patients from the control group (patient HE210023 being positive for 2 different bacteria). These latter patients were positive in 4/4, 3/4, 2/4 and 1/4 positive wells, respectively for 2, 2, 3 and 18 nasopharyngeal swabs (Table S2). *Criblamydiaceae* species were recovered from 4 patients with pneumonia (all with 1/4 positive well) and 1 patient from the control group (4/4 positive wells). *Simkaniaceae* species were found in 5 patients (3 control patients and 2 children with pneumonia). Finally, *Rhabdochlamydiaceae* species were identified in 1 case of pneumonia and 2 control subjects. Thus, 17 and 20 children with and without pneumonia respectively, were positive for a *Chlamydia*-related bacterium. In addition, a sample taken from a control subject could not be affiliated in any range of family-level lineage (unclassified *Chlamydiales*).

All 60 non inoculated Copan swabs were found negative with the pan-*Chlamydiales* PCR, demonstrating that the positive samples were not false positive. Furthermore, no absence

of the internal amplification control was observed, excluding false negative results due to PCR inhibitors.

DISCUSSION

In this work, we developed a new *Chlamydiales*-specific PCR that proves to be specific to the *Chlamydiales* order, to be sensitive for at least 5 DNA copies per reaction of the positive control (with an efficiency of 75%) and to be highly reproducible. Moreover, its application to clinical samples taken from children with and without pneumonia demonstrated the common exposure of humans to various *Chlamydia*-related bacteria. This new PCR showed a broad range of targeted species since it detected the 15 different chlamydial strains tested and the DNAs of 36 never described species-level lineages (<97% similarity of the 16S rDNA sequence) of the Chlamydiales order (>80% similarity of the 16S rDNA sequence) (16, 25) present in nasopharyngeal swabs samples (Supplementary Table S2). Furthermore, this new PCR could detect chlamydial DNA from samples of various origins (Table 3).

Previous classical pan-*Chlamydiales* PCRs have already been developed (10, 36, 43) but they detected from 1000 DNA copies compared to real-time PCRs that can detect about 200 to 1000-fold less DNA copies per reaction. This higher sensitivity is likely due to the shorter reads (about 200 bp) and the read-out thank to a fluorescent Taqman probe. Considering this high sensitivity, DNA extraction, real-time PCR and sequencing reactions were processed in separate rooms to avoid contaminations between samples. In addition, automated DNA extraction located outside from our research laboratory was preferred. Moreover, since no sequence obtained showed more than 97% similarity with bacteria grown in our laboratory, a contamination may not explain the obtained results. The

sequencing of most positive samples was possible and results were informative at the family-level. A previous study using short sequences of *Chlamydiales* also successfully identified strains at the family-level with similar length sequences (140 to 195 bp) (43). Further identification, at the species-level, may be performed using complementary methods (PCR targeting a more discriminative core gene such as *rpoB* or *gyrA*).

As previous studies on nasal and/or nasopharyngeal samples have already allowed the recovery of *Chlamydia*-related bacteria or the amplification of DNA of these obligate intracellular bacteria (1, 12, 15, 36), we chose similar samples for the first application of the new PCR. The sequencing results on these nasopharyngeal swabs confirmed previous studies on the occurrence of *Chlamydia*-related bacteria in nasal mucosa of healthy individuals (1). They also clearly showed that the biodiversity of *Chlamydia*-related bacteria is far from being established: among the 48 sequences, 36 were from putative new species, when considering the Everett cut-off of < 97% 16S rRNA similarity to define species-level lineages and all were belonging to the *Chlamydiales* order (>80% similarity of the 16S rDNA sequence) (16, 23, 24). Thus, our work clearly demonstrates the common exposure of children to different *Chlamydiales*, since around 11.4% of patients were positive with the new PCR. When a *Chlamydiaceae* species was amplified, it was generally from a sample taken from a child with pneumonia (6/7). Noteworthy, DNA of *Criblamydiaceae* were also mainly amplified from patients with pneumonia (4/5), whereas other *Chlamydia*-related bacteria were amplified from nasopharyngeal swabs taken from both children with and without pneumonia. Thus, although our study demonstrated a common exposure to *Parachlamydiaceae*, (amplified from 5.9% of all samples), these were not over-expressed in the pneumonia group. Nonetheless, since the sequencing does not allow identification at

273 the species level, a significant correlation with a given species may not be excluded.
274 Similarly, our work did not bring any argument in favor of an association of *Simkaniaceae*
275 with pneumonia in children. This was somehow expected, since initial studies that
276 suggested an association of *Simkania negevensis* with diverse respiratory infections in
277 children (15, 18-20, 22, 32, 33, 35) were not confirmed in more recent works (34). Further
278 research is now needed to specify the pathogenic role of each representing species in the
279 *Chlamydiales* order.

280 In conclusion, this work provides a new diagnostic approach to specify the biodiversity and
281 pathogenic role of *Chlamydia*-related bacteria and highlights the common exposure of
282 children to *Parachlamydiaceae*.

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Figure 1: Reproducibility and efficiency of the new real-time PCR. Inter and intra-run reproducibility was performed between 12 different runs representing 72 duplicates of positive control. (A) Inter-run variability. (B) Bland-Altman graph represents the intra-run variability between duplicates and a bias of 0.36 was calculating as well as the 95% limit of agreement shown by the dashed line. (C) Efficiency was evaluated with 20 replicates of 5 different plasmid control concentration (50, 20, 5, 1 and 0.5 copies per reaction).

Supplementary figure S1: the graph shows cycle threshold (Ct) values according to the percentage of positive wells obtained for nasopharyngeal samples. The dashed line represents the mean Ct (36.3) obtained for 24 replicates of 5 DNA copies of the positive plasmid control per reaction (Fig.1A). The majority of clinical samples were detected at Ct values >36.3, corresponding to <5 DNA copies per reaction, for which the efficiency of the PCR decreases.

428

429 Table 1: Bacterial and amoebal species used to test the specificity.

BACTERIAL SPECIES	Source/strain
*Bacteroides fragilis**	ATCC25825
*Escherichia coli**	ATCC 25922
*Haemophilus influenzae**	ATCC 49247
Legionella pneumophila	Clinical specimen
Mycoplasma pneumoniae	Clinical specimen
*Pseudomonas aeruginosa**	ATCC 27853
*Staphylococcus aureus**	ATCC 25923
Streptococcus mitis	ATCC 6249
*Streptococcus pneumoniae**	Clinical specimen
AMOEBAL SPECIES	Source/strain
Acanthamoeba castellanii	ATCC 30010
Acanthamoeba comandoni	Strain WBT
Dictyostelium discoideum	DH1-10
Hartmannella vermiformis	ATCC 50237

430 *Bacterial DNA used in the competition test with *Pr. naegleriophila* strain KNic.

Table 2: Chlamydial DNA used to evaluate the range of the new PCR

Chlamydial species	Source/strain
Chlamydia abortus	Strain S26/3[a]
Chlamydia pecorum	Strain W73 [b]
Chlamydia pneumoniae	Strain K6 [c]
Chlamydia psittaci	Strain T49/90 [d]
Chlamydia suis	Strain S45/6[a]
Chlamydia trachomatis	Clinical specimen
Criblamydia sequanensis	Strain CRIB-18
Estrella lausannensis	Strain CRIB-30
Neochlamydia hartmannellae	ATCC 50802
Parachlamydia acanthamoebae	Strain Hall's coccus
Parachlamydia acanthamoebae	ATCC VR-1476 (strain Bn9)
Candidatus *Protochlamydia amoebophila*	ATCC PRA-7 (strain UWE25)
Protochlamydia naegleriophila	Strain KNic
Simkania negevensis	ATCC VR-1471
Waddlia chondrophila	ATCC VR-1470

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

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

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

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

438 Table 3: Analysis of samples from various origins by the pan-*Chlamydiales* PCR in

439 comparison with the *C. pneumoniae* PCR (A) and the *C. trachomatis* PCR (B).

440 A

	C. pneumoniae		Pan-*Chlamydiales*	
Samples	+	-	+	-
Nasopharyngeal swabs	1	1 [1/0]	1	1
Bronchoalveolar lavages	0	6 [2/1]	0	6
Bronchial aspirates	0	4 [0/2]	0	4
Sputa	1	2 [2/0]	1	2
Sub-total	2	13	2	13
Total	15		15	

441 Superscript numbers indicate the number of positive sample for *Mycoplasma pneumoniae*

442 and the number of positive sample for *Legionnella pneumophila* (*M.pn./L. pn.*), respectively

443 B

	C. trachomatis		Pan-*Chlamydiales*	
Samples	+	-	+	-
Vaginal or cervical swabs	14	15	14	15
Anorectal swabs	14	0	12	2**
Urethral swabs	1	0	1	0
Eye swabs	1	1	1	1
Urines	32	33	31(+1°)	32(+1*)
Ascitis liquid	1	1	0	2*
Sub-total	63	50	60	53
Total	113		113	

444

445 * 1 or **2 sample(s) positive for *C. trachomatis* but negative with the pan-*Chlamydiales* PCR;

446 ° 1 sample negative for *C. trachomatis* but positive with the pan-*Chlamydiales* PCR.

447 Table 4: Sequencing results of nasopharyngeal samples from the pneumonia group positive with the new pan-*Chlamydiales*

448 PCR

Patient no.	Sex	Age (years)	Signs and symptoms	Other etiology	Underlying condition(s)	% 16S rRNA gene homology with most similar GenBank sequence (corresponding family)
GE10160	F	3.7	39.0°C, cough, thoracic pain, DRS	-	coeliakie	100% *Chlamydia pneumoniae* LPCoLN (Ch)
GE10097	F	2.7	40.6°C, cough, DRS	-	-	100% *Chlamydia pneumoniae* LPCoLN (Ch)
VS30014	M	12.4	38.9°C, cough	-	-	100% *Chlamydia pneumoniae* LPCoLN (Ch)
VS30030	F	12.2	39.5°C, cough	-	-	100% *Chlamydia trachomatis* D-LC (Ch)
GE10098	F	8.0	38.0°C, cough	-	-	100% *Chlamydia pneumoniae* CWL029 (Ch)
GE10014	M	7.6	39.5°C, cough	*M. pneumoniae*	-	94% Uncultured *Neochlamydia* sp. LTUNC09656 (P)
GE10159	M	3.7	40.0°C, cough	-	-	100% *Chlamydia pneumoniae* LPCoLN (Ch)
GE10169	M	5.6	40.0°C, cough, thoracic pain, tachypnea	-	Bronchodysplasia, premature birth (28 weeks)	97% Candidatus *Rhabdochlamydia porcellionis* (R) 91% *Chlamydiales* bacterium cvE38 (S)
GE10179	F	3.6	Dyspnea	*S. pneumoniae* H1N1 virus	-	Sequencing failed
HE20032	M	1.5	41.6°C, cough, DRS	-	-	94% Uncultured Candidatus *Protochlamydia* sp. clone CN823 (P)
VS30003	M	5.8	39.5°C, cough, DRS	-	-	95% Uncultured *Chlamydiales* bacterium clone P-5 (P)
GE10027	M	9.8	38.1°C, cough, DRS	*M. pneumoniae*	asthma	92% Uncultured soil bacterium clone 530-2 (Cr)
GE10036	M	1.6	38.0°C, cough, DRS, tachypnea	*S. pneumoniae*	-	95% Uncultured bacterium clone F5K2Q4C04JDDHX (P)
GE10047	M	2.6	39.5°C, cough, DRS, tachypnea	-	-	92% *Criblamydia sequanensis* (Cr)
GE10072	F	4.8	39.5 °C	HMPV A	-	Sequencing failed
GE10147	M	13.8	38.2°C, cough, DRS	*S. pneumoniae*	-	95% Uncultured bacterium clone FW1013-189 (P)

GE10193	F	5.1	38.9°C, cough, tachypnea	-	-	92% *Chlamydiales* bacterium cvE38 (S)
HE20008	F	3.3	38.3°C, cough, tachypnea, DRS	HUK1	-	93% *Estrella lausannensis* strain CRIB 30 (Cr)
HE20028	F	1.1	39.5°C, cough, DRS, tachypnea	HRSV A	-	94% Uncultured *Chlamydiales* bacterium clone P-5 (P)
HE20036	M	1.4	40.0°C, DRS	-	-	96% *Chlamydiales* bacterium cvE21 (E6)
HE20074	M	4.5	38.1°C, cough, DRS	-	-	94% *Criblamydia sequanensis* (Cr)
VS30007	M	6.6	38.7°C, cough, DRS	-	-	95% Candidatus *Metachlamydia lacustris* strain CHSL (P)
VS30013	M	6.4	38.5°C, cough, tachypnea	-	-	94% Uncultured *Chlamydiales* bacterium clone P-9 (P)
VS30044	M	3.8	40.4°C, cough	-	-	95% Uncultured *Chlamydiales* bacterium clone P-7 (P)
VS30055	F	4.1	38.5°C, cough, tachypnea	-	-	92% Candidatus *Metachlamydia lacustris* strain CHSL (P)

449 F= female, M = male; DRS = distress respiratory syndrome;

450 Ch= *Chlamydiaceae*, P = *Parachlamydiaceae*, R = *Rhabdochlamydiaceae,* S = *Simkaniaceae*, Cr = *Criblamydiaceae*, E6= novel E6-

451 lineage.

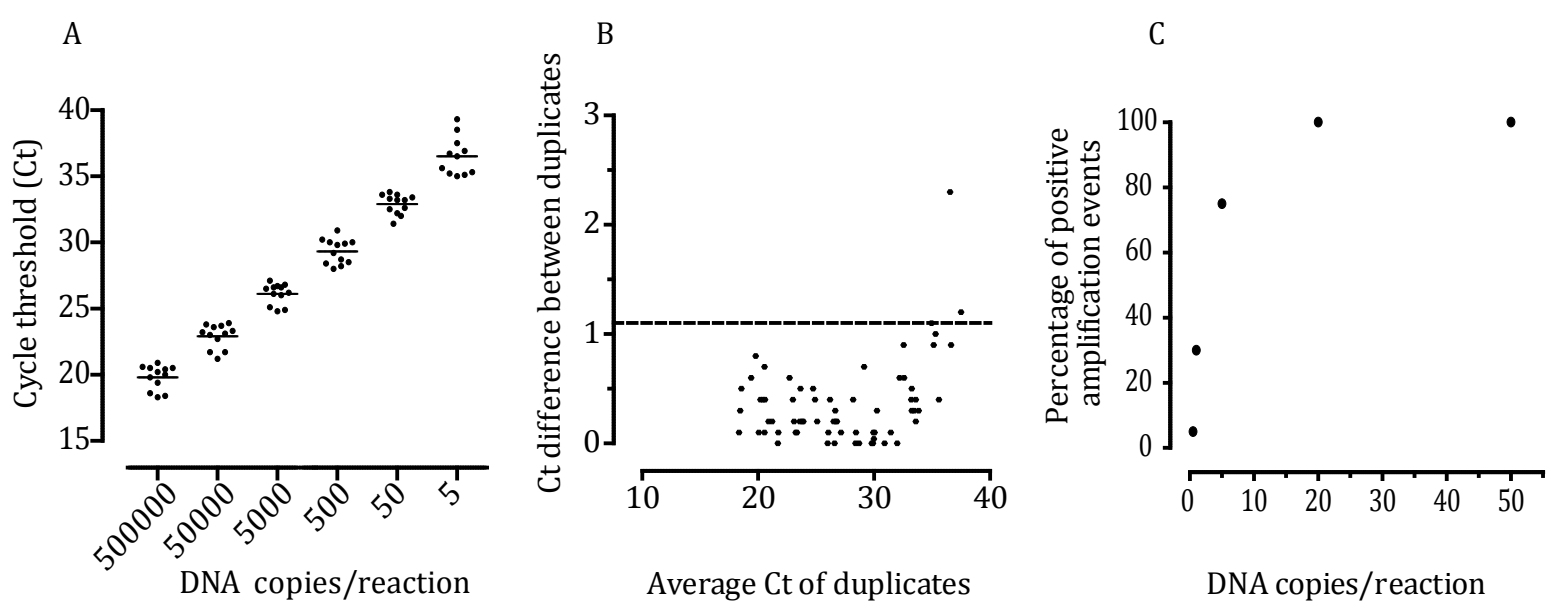

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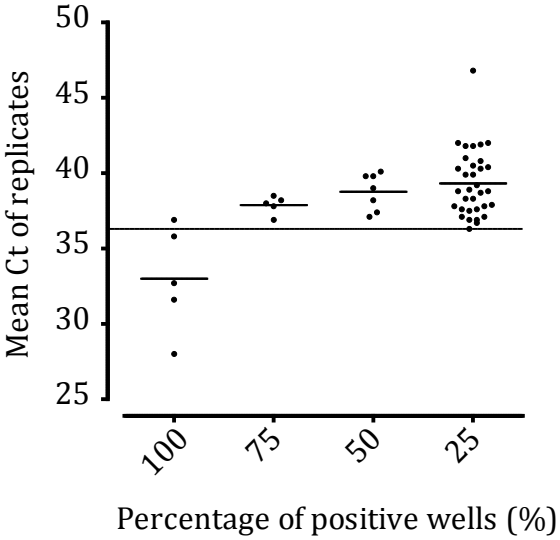
Table S1: Sequencing results of 7 samples from the retrospective study positive with the new PCR

Sample	Sequence (bp)	Threshold cycle (Ct) with the new PCR: mean ±SD	Expected result of the sequencing	% 16S rRNA gene sequence similarity with most similar GenBank sequence (Accession no.)
Sputa	183	36.1	CP +	100% *Chlamydia pneumoniae* LPCoLN (CP001713.1)
Nasopharyngeal swab	183	35.5±0.7	CP +	100% *Chlamydia pneumoniae* LPCoLN (CP001713.1)
Cervical swab	192	23.6±2.9	CT +	100% *Chlamydia trachomatis* D-LC (CP002054.1)
Anorectal swab	151	39.0±0.2	CT +	100% *Chlamydia trachomatis* D-LC (CP002054.1)
Urine	192	35.0±0.8	CT +	100% *Chlamydia trachomatis* D-LC (CP002054.1)
Cervical swab	206	29.3±0.3	CT +	100% *Chlamydia trachomatis* D-LC (CP002054.1)
Urethral swab	141	31.7±0.5	CT +	99% *Chlamydia trachomatis* D-LC (CP002054.1)
Urine	190	41.3	CT -	94% Uncultured bacterium clone FW1013-189 (EF693090.1)

CP = *Chlamydia pneumoniae*, CT = *C. trachomatis.*



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