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Methods for real-time PCR-based diagnosis of *Chlamydia pneumoniae*, *Chlamydia psittaci* and *Chlamydia abortus* infections in an opened molecular diagnostic platform

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Summary

The advances in molecular biology of the last decades have dramatically improved the field of diagnostic bacteriology. In particular PCR-based technologies have impacted the diagnosis of infections caused by obligate intracellular bacteria such as pathogens from the *Chlamydiacae* family. Here, we describe a real-time PCR-based method using the Taqman technology for the diagnosis of *Chlamydia pneumoniae*, *Chlamydia psittaci* and *Chlamydia abortus* infection. The method presented here can be applied to various clinical samples and can be adapted on opened molecular diagnostic platforms.

Key words: *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Chlamydia abortus*, molecular diagnostic, real-time PCR, DNA extraction, Taqman

1. Introduction

Chlamydiacae are obligate intracellular bacteria, among which several species are pathogenic towards humans and can cause a broad range of diseases. *Chlamydia trachomatis* is involved in urogenital infection as well as ocular, joint and oropharyngeal infections. *Chlamydia pneumoniae* and *Chlamydia psittaci* are primarily associated with community-acquired pneumonia (CAP). *C. pneumoniae* have been essentially associated with infections in humans [1] but some studies suggest an association with other mammals such as koalas [2-8]. *C. pneumoniae* might also be a causative agent of an asthma-like syndrome in children [9]. *C. psittaci* is the etiologic agent of a respiratory zoonosis transmitted by birds, in particular parrots and parakeets, but also chicken from the food industries as well as feral pigeons [2]. *C. abortus* is genetically closely related to *C. psittaci* but displays a distinct animal and tissue tropism as it can colonize the placenta of cows, goats, cattles, pigs and horses, which can lead to abortion. *C. abortus* can be transmitted to humans, for instance through the exposure to infected animal abortive tissues, with the same outcome for pregnant women [2].

The diagnosis of infections due to intracellular bacteria has been dramatically improved by PCR-based methods developed in the last decades. This is particularly true for the diagnosis of *C. trachomatis,* for which several commercial systems have been developed. The laboratory of molecular diagnostic of the Institute of Microbiology of the Lausanne University Hospital (Lausanne, Switzerland), developed a molecular diagnostic platform recently described by Greub and colleagues, which allows one to perform multiple PCRs simultaneously targeting different pathogens, thanks to the standardisation of the parameters (i.e. amplicon length, and primers and probes Tm) of the reactions [10]. This molecular platform based on the Taqman-probes technology (Applied Biosystems) allows for performance of up to 91 different PCR reactions corresponding to 69 pathogens and/or resistance genes in a single microplate [10]. Among them are a real-time PCR for the detection of *C. pneumoniae* [11] and a real-time duplex PCR for the detection of *C. psittaci* and *C. abortus* infection from various clinical samples (Table 1), both based on the Taqman probe technology [12]. The *C. pneumoniae* PCR is based on the *pst1* gene and the *C. psittaci*-*C. abortus* duplex PCR was designed as follows: 1) PCR1 targets a DNA sequence of the 16S–23S rRNA operon allowing the detection of both *C. psittaci* and *C. abortus* and 2) PCR 2 targets the coding DNA sequence CPSIT_0607 so far unique to *C. psittaci* [12]. In this chapter, the methods to achieve the *C. pneumoniae* PCR and the *C. psittaci*-

C. abortus duplex PCR on an opened molecular diagnostic platform are detailed. For this specific chapter, part of the samples' processing methods and infrastructure descriptions have been extracted from the accredited documentation of the Laboratory of Diagnostic of the Institute of Microbiology of the University of Lausanne.

2. Materials

2.1 Laboratory organization

All the procedures should be carried-out according to molecular diagnostic principles aimed to avoid contaminations with microorganisms or nucleic acids (See Note 1).

2.2 Sample processing

1. N-acetyl cysteine solution: in a 50 ml conical tube, containing 1.0 g of N-acetyl cysteine, add 50 ml of Tris-Sodium-Citrate-di-hydrate buffer. The solution should be used the same day.
2. Molecular biology grade PBS: DNase-Free, RNase-Free, does not contain detectable amounts of nucleic acid or any nucleic-acid extractions' compatible molecular biology grade solution.

2.3 Material for DNA extraction

1. Several automated instruments exist for DNA extraction, in the Institute of microbiology, nucleic-acid extraction is achieved with the MagNA Pure 96^{R} instrument (Roche): an automated system for the extraction of nucleic-acid from bacteria (as well as other microorganisms such as viruses) using a technology based on magnetic glass particles. The MagNA Pure 96^{R} instrument reagents are those provided by the manufacturer. This nucleic-acid extraction instrument has been associated with a liquid handling distribution system, the STARlet^{R} instrument (Hamilton^{R}) [13] (See Note 2).

2. Molecular biology grade PBS: DNase-Free, RNase-Free, does not contain detectable amounts of nucleic acid or any nucleic-acid extractions' compatible molecular biology grade solution.

2.4 Assembly of the PCR plate and amplification

1. TaqMan Universal Master Mix (Applied Biosystems).
2. Primers and probes (Table 1).

3. Molecular biology grade water: DNase- and RNase-free water, which does not contain detectable amounts of nucleic acid.
4. Positive control. The positive controls consist of synthetic plasmids containing the exact PCR amplicon [12,11].
5. PCR instrument (see Notes 3 and 4).

3. Methods

3.1 Sample processing

In clinical practice, the detection of *C. pneumoniae*, *C. psittaci* and *C. abortus* can be achieved from a broad range of samples (Table 2). Depending on the nature of the sample (liquid, viscous or solid) a specific processing will be required either to concentrate (liquid samples) or to homogenize (viscous or solid samples) the specimen.

1. Introduce the lab request into the laboratory information system (LIS) to generate the barcode labels necessary to manage tubes during the analytic process (See Note 5).
2. For each sample, label three screw cap tubes (2ml) with the barcode as follows: one "native" tube, one "native-aliquot" tube and one "DNA tube".
3. Under the laminar flow dedicated for molecular diagnostics in the "specimen-receiving laboratory," distribute the sample into the native-aliquot tube and the native tube. The native tube will be frozen at -80°C and kept as backup in case of need. If necessary several native tubes can be stored; a suggestion for biopsies would be to store both the native samples and the processed samples in distinct tubes. The aliquot-native tube together with the DNA tube will be transported to the molecular diagnostic laboratory and used for nucleic acids extraction. Viscous samples such as respiratory secretions should be homogenized by liquefaction where as solid samples such as biopsies and fragments should be crushed as described below.
4. Liquefaction of viscous secretions: respiratory secretions (sputum, bronchial aspirate, stomach tube) for the detection of *C. pneumoniae* and *C. psittaci* can be too viscous to be homogenized by simple vortexing. Liquefaction can be achieved using N-acetyl-L-cysteine, a reducing substance having free thiol groups (-SH), to reduce specimen

viscosity by breaking the disulfide bonds of glycoproteins that constitute the bronchial mucus. Any other viscous fluids shall also be liquefied. To do so, visually control the viscosity of the sample; if liquefaction is necessary, transfer a suitable amount of the sample in a 15 or 50 ml conical tube using a single use plastic Pasteur pipette, under the hood with laminar flow. Add an equal amount of N-acetyl-L-cysteine solution. A larger amount of N-acetyl-L-cysteine solution can be needed for very sticky samples. Vortex and visually check the liquefaction of the sample and if necessary, leave the sample on a rotor for up to 30 minutes. At the end of the incubation, visually check the liquefaction of the sample and centrifuge the sample 30 minutes at 3000 *g* then remove the supernatant using a pipette with filter tips, leaving 1 to 2 mL of liquid in which the pellet will be resuspended. Transfer the amount necessary for nucleic acids extraction (more than 200 µl) into the tube labelled as aliquot-native" (See Note 6), and keep the rest in the tube labelled as "native". Store the native tube at -80°C in the core specimen receiving laboratory and transport the aliquot-native tube and the DNA tube to the molecular diagnostic laboratory.

5. Crushing of solid specimens. Solid samples such as biopsies and fragments that cannot be homogenized either using the vortex or by liquefaction should be crushed as follows. Under the hood with laminar flow put the little pieces in the crushing device with part of the solution of the native sample (if any) or add a solution adapted to the DNA extractor (PBS) using a pipette with filtered tips (see Note 7). If the specimen is too big, take a piece of it with sterile forceps and place it in a sterile Petri dish to cut it into several small pieces. Crush a piece of the specimen and keep the rest in reserve. Start the crushing device. Generally, the fragments are successfully crushed, if not, this procedure is still sufficient to release microorganisms from the specimen by compression. In this case, avoiding some remaining large fragments, transfer the crushed sample (volume) in the aliquot-native tube, and the rest in the native tube for storage at -20°C. If necessary add some molecular biology grade water into the aliquot-native tube to reach the minimum volume suitable for nucleic acids extraction. Vortex the tube. Transport the aliquot-native tube and the DNA tube to the molecular diagnostic laboratory.

3.2 DNA extraction

1. Using the STARlet^{R} liquid handling instrument, transfer 200 ul of the sample tube in the extraction plate of the MagNA Pure 96^{R} instrument.
2. Extraction control (EC). The EC corresponds to a tube submitted to the same extraction protocol that the clinical specimen but in which the volume of the clinical sample is replaced by an equal volume of a solution adapted to the DNA extractor (PBS). An extraction control is needed for each run of extraction (see Note 8). The extraction control must be negative when the PCR specific for the pathogen (s) tested is (are) made.
3. Transfer the 96-well microplate into the MagNAPure 96^{R} instrument and start the nucleic acids extraction program according to the user manual. Nucleic acids can be eluted either in 50µl or 100µl of elution buffer depending on the chosen program and are maintained at 4°C (See Note 9).

3.3 Preparation of the PCR controls

1. Negative control of PCR. Each run of amplification should contain a PCR negative control that consists of the extraction control used as a template. The negative control is used to test the reactivity of the component of the reaction mixture. It should not be contaminated with target DNA and should not allow non-specific amplification.
2. Positive control. Positive controls consist of synthetic plasmid DNA containing the target sequence of the PCRs [12,11] (See Note 10). For each run of PCR, three reactions with three dilutions of the positive controls containing 10, 10^2 and 10^3 DNA copies per reaction should be added. They will serve both to generate the standard curve that will be used for the quantification of the positive control and to determine the sensitivity of the reaction based on the positive amplification, the reaction containing 10 copies of DNA.
3. Inhibition control. The presence of PCR inhibitors should be tested for each sample. To do so the inhibition control reaction consists of a reaction in which 200 copies of the positive control is added to the reaction mixture containing the DNA specimen to be tested [10].

3.4 Preparation of the PCR mix, design and assembly of the PCR microplate and amplification

1. Design of the Taqman PCR plate. This can be achieved using dedicated software such as the SDS 2.4.1 software (Applied Biosystems) allowing the design of either 96 or 384 well PCR plates **[10]**. The same final volume (20 µl) is convenient for both type of plate. It is strongly recommended to do each analysis in duplicate (or even in triplicate). When an internal control is not used, an additional well for the inhibition control is needed, if not using internal inhibition control. Moreover, there is a need for at least one well for the inhibition control, one well for the negative control and 3 wells for the standard curve. A standard curve is required for each PCR. One regression curve is required for the PCR1 and another for the PCR2 [12].
2. In the DNA-free laboratory, prepare the PCR mix. A single mix is needed for the detection of *C. psittaci* and *C. abortus*. In a final volume of 20 µl, add 5 µl of the extracted DNA, the forward and reverse primers and the probes at the final concentration indicated in table 2. The inhibition control reaction consists of the same reaction in which, 200 copies of the control plasmids are added. As an extraction negative control the DNA is replaced by the same volume of the extraction control. As a negative control of the PCR, the DNA is replaced by molecular grade water.
3. Assembly of the PCR plate. The assembly of the PCR plate can be done manually for 96 well plates (See Note 11) or using automated instruments for 384 well plates [10]. The reactions are achieved in a final volume of 20 µl with 5 µl of DNA sample [12,11].
4. Amplification. Run the ABI 7900 instrument or similar thermocycler using the following cycling conditions: 2 min at 50°C, 10 min at 95°C followed by 45 cycles of 15s at 95°C and 1min sec at 60°C.

3.5 Interpretation of the results

The SDS 2.4.1 software is used for analysis and interpretation of the results.

The results (qualitative and quantitative) are then checked and introduced into the LIS system.

At the end of the process the final results are validated by a clinical microbiologist.

1. Analyze the results of the positive controls with adequate software [10].
2. Internal quality control. For an analysis to be valid: a) the run should pass the internal quality control and b) the positive controls 10 copies must be detected.

3. Negative controls. Control that there is no amplification in the negative control reaction.
4. Inhibition control. Control that there is amplification in the inhibition control tube containing 200 copies of the positive control plasmid (at least of 50 copies).
5. Positive result. A result is positive if the fluorescence reaches the threshold automatically set by the software or manually set by the user according to the instrument.
6. Interpretation of the *C. psittaci-C. abortus* PCR (See Note 12).
7. If you suspect a contamination, when for instance only 1 reaction out of 3 reactions is positive or when amplification occurs in the extraction control or in the PCR negative control, you can follow the procedure described by Greub and colleagues for these situations [10].

4. Notes

Note 1

As described by Greub and colleagues it is important to organize the laboratory of molecular diagnostic in different rooms/spaces corresponding to pre-amplification and post-amplification area **[10]**. Moreover, the processing of clinical samples in the laboratory where the samples are received should be achieved under a laminar flow dedicated for molecular diagnostics that is distinct from laminar flows dedicated for conventional culture based microbiology diagnostics. Sterile samples should also be processed in dedicated laminar flows. The use of disposable lab coats, gloves and pipettes with filter tips is recommended. Thus, we recommend the following infrastructures and instruments: **A)** the sample reception laboratory should be equipped with a hood with laminar flow and UV, a vortex, a crushing instrument, a centrifuge, disposable lab coats and gloves, pipettes and filter tips, 2ml screw cap micro-tubes, sterile forceps and sterile Petri dishes; **B)** the nucleic acids' extraction laboratory should also be equipped with a hood with laminar flow and UV, a vortex, pipettes and filter tips, disposable lab coats and gloves without mineral powders such as talc to prevent any deposition in the extraction tubes; **C)** the PCR master-mix should be prepared in a DNA free laboratory equipped with a hood with UV but without laminar flow; for all the post extraction area, hood with laminar flow should be prohibited in order to avoid the deposition of nucleic acids (NA) in opened tested tubes. In addition, the following instruments should be available in this laboratory: a vortex, a micro-centrifuge, micro-tubes (0.2, 0.7 and 1.5 ml), 2ml screwed tube, pipettes and filter tips (0-10, 2-

20, 20-200 et 200-1000 µl); **D)** the positive control should be prepared and stored in a "DNA laboratory" equipped with a hood with UV but without laminar flow, a vortex, a micro-centrifuge, micro-tubes, pipettes and filter tips; **E)** It is recommended to assemble the PCR plate a dedicated pre-amplification laboratory different from the positive control laboratory.

Note 2:

If processing a large number of samples, it is recommended to select automated distribution systems that can recognize bar-coded tubes.

Note 3:

The assembly of the PCR plate can be achieved manually for 96 well plates or alternatively can be done by automated instruments to increase the precision and to avoid mistakes, especially when preparing 384 well plates **[10]**.

Note 4:

The PCR conditions described herein for the detection of *C. pneumoniae, C. psittaci* and *C. abortus* have been optimized for the PCR instrument ABI 7900 HT (Applied Biosystems) **[12]**. The reagent concentrations and the amplification program should be adapted if using other instruments **[10]**.

Note 5:

Tubes management is crucial all along the analytic process to avoid major errors such as inversions or contaminations. We strongly recommend the use of barcoded tubes.

Note 6:

If using an automated system such as the STARlet^{R} instrument (Hamilton^{R}), do not forget to provide the dead volume of the device.

Note 7:

To avoid the dilution of the specimen, which could negatively impact the sensitivity of the PCR, do not add too much solution for the crushing of the sample.

Note 8:

We recommend placing the extraction control at the end of the series where contamination are more expected to occur rather than at the first position of a series.

Note 9:

It is recommended to store the DNA sample at -80 ° C.

Note 10:

The concentration (DNA copies per ml) of the positive control should be precisely determined. The stock solution of the positive control should be stored in a separate, dedicated room.

Note 11:

The assembly of the PCR plate can be achieved manually especially for 96 well plates. In the DNA free laboratory prepare the PCR mix without the DNA. Place a 96-well micro-plate on a chilled metal rack and distribute the PCR master mix in the 96-well plate according to the Taqman plate set-up. Add the molecular grade water to the corresponding wells (*i.e* in the negative controls) and transfer the plate with the chilled metal plate in the assembly laboratory. In the assembly laboratory, add the DNA samples starting with the extraction control to avoid any contamination at this stage. Close the tubes when the DNAs of a patient are pipette in order to avoid contamination by aerosols. Repeat this for all the patients' DNAs. When all the patients' DNAs are added, cover the corresponding wells and carefully add the positive control in the wells corresponding to the "inhibition control". It is important to avoid any contamination of the patients' test-tube with the positive controls which would lead to false positive results. Exit the room with the plate in a chilled metal rack and transfer it to the amplification room where the thermocycler is located.

Note 12:

As described in Opota et al 2015, if both PCR are positive (PCR1 and PCR2) in a respiratory sample, this indicates the presence of *C. psittaci* DNA [12]. Samples positive for PCR1 and

negative for PCR2 can be considered as positive for *C. psittaci* for respiratory specimens and positive for *C. abortus* for genital specimens.

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370 **Tables**

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Table 1: Primers and probes

Pathogen	Oligonucleotide name	Target gene	Sequence (5'-3')	Modification, fluorochrome	Amplicon lengh	Final Concentration (µM)
C. pneumoniae	CPTM1	*pst1* gene	CATGGTGTCATTCGCCAAGT	-		0.2
	CPTM2	*pst1* gene	CGTGTCGTCCAGCCATTT TA	-		0.2
	CP	*pst1* gene	TCTACGTTGCCTCTAAGAGAAAACTTCAAGTTGGA	3'-VIC, 5'-TAMRA	82	0.1
C. psittaci and *C. abortus*	CPSI_F	16S-23S operon	AAGGAGAGAGGCGCCCAA	-		0.35
	CPSI_R_LNA	16S-23S operon	CAA[C]CTAGTCAAACCGTCCTAA	LNA		0.35
	CPSI_P_MGB	16S-23S operon	ACTGGGATGAAGTCGTAAC	FAM, DQ	133	0.2
C. psittaci	CPSI_00F	CDS CPSI_0607	AGCATTAGCCAGCGCTTTAGA	-		0.35
	CPSI_00R_147C/G	CDS CPSI_0607	TCTCTGAGCAAAAAC/GACTGCGT	-		0.35
	CPSI_00P_MGB	CDS CPSI_0607	ACAAAGACCTGGCGAGTA	VIC, DQ	118	0.2

LNA = Locked nucleic acid, MGB = Minor groove binder, FAM = 6-carboxy-fluorescein, VIC = TaqMan VIC reporter dye, DQ = Dark quencher, BHQ = Black hole quencher

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Table 2. Clinical specimens and pathogens generally used for the diagnosis of *Chlamydia* infections

	Sample type	*C. pneumoniae*	*C. psittaci*	*C. trachomatis*	*C. abortus*
Oral	Mouth swab			x	
Respiratory specimen	Nasal swab	x	x		
Respiratory specimen	Nasopharyngeal seretions	x	x		
Respiratory specimen	Sputum	x	x		
Respiratory specimen	Bronchial secretion	x	x		
Respiratory specimen	Bronchoalveolar lavage	x	x		
Urogenital specimen	Uterus and urethral smear			x	x
Urogenital specimen	Urethral swab			x	
Urogenital specimen	Prostate biopsies			x	
Urogenital specimen	Fragment of placenta			x	x
Anal	Anal swab			x	
Osteo-articular	Joint fluid			x	
Osteo-articular	Prosthetic fragment			x	
Vascular	Drain fluid from aortic valve				
Animal specimen	Bird spleen		x		
Animal specimen	Birds choanal or cloacal swabs		x		
Animal specimen	Fragment of sheep placenta				x

Non-comprehensive list

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