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Detection and Differentiation of Chlamydiae by Nested PCR

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1. Introduction

1.1. Characteristic Features and Importance of the Agents

Chlamydiae are obligate intracellular prokaryotes with a hexalaminar cell wall that, in contrast to other gram-negative bacteria, contains no peptidoglycan. As a major antigenic constitutent, their outer membrane contains a 10-kDa lipopolysaccharide (LPS) with a trisaccharide epitope specific for the family *Chlamydiaceae* ***(1)***. Another characteristic antigenic component is the major outer membrane protein (MOMP), a cysteine-rich protein of approx 40 kDa representing approx 60% of the weight of the outer membrane. This molecule harbors several genus- and species-specific antigenic determinants in the conserved regions and serovar-specific epitopes in variable domains ***(2)***.

A unique feature of chlamydiae is the biphasic developmental cycle, in the course of which two distinct morphological forms emerge. At the extracellular stage, the smaller infectious and metabolically inactive elementary bodies are prevailing, whereas the larger metabolically active and self-replicating reticular bodies reside in vacuole-like cytoplasmic inclusions of the host cell.

Traditionally, the genus *Chlamydia* (*C.*) comprised four species, i.e., *C. trachomatis*, *C. psittaci*, *C. pneumoniae*, and *C. pecorum*. However, a large amount of new DNA sequence data led Everett et al. ***(3)*** to reassess genetic relatedness and propose taxonomic reclassification. According to this proposal, the family *Chlamydiaceae* consists of two genera, *Chlamydia* and *Chlamydophila*, with a total of nine largely host-related species. The two classification schemes are summarized in **Table 1**.

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Table 1
Comparison of Old and New Taxonomic Classification Schemes for the Family *Chlamydiaceae*

Traditional species	New species ***(3)***	Host specificity[a]
Chlamydia trachomatis	*Chlamydia trachomatis*	human
	Chlamydia muridarum	mouse, hamster
	Chlamydia suis	swine
Chlamydia psittaci	*Chlamydophila psittaci*	birds, ruminants, horse
	Chlamydophila abortus	sheep, other rumi nants, swine, birds
	Chlamydophila caviae	guinea pig
	Chlamydophila felis	domestic cat
Chlamydia pecorum	*Chlamydophila pecorum*	cattle, sheep, goat, swine, koala
Chlamydia pneumoniae	*Chlamydophila pneumoniae*	human, koala, horse, amphibians

[a] Hosts are given according to present knowledge. As species-specific methods of identification will be used to a greater extent in the future, further widening of the host range can be expected in some cases.

The group of chlamydial species includes agents of important animal and human diseases. Chlamydiae are very wide spread in many host organisms, but not nearly all carriers develop symptoms of disease.

Avian strains of *C. psittaci* (new classification: *Chlamydophila psittaci*) can cause psittacosis, a systemic disease in psittacine birds of acute, protracted, chronic, or subclinical manifestation ***(4,5)***. The analogous infection in domestic and wild fowl is known as ornithosis. Avian chlamydiosis is transmissible to humans, the symptoms being mainly nonspecific and influenza-like, but severe pneumonia, endocarditis, and encephalitis are also known.

Enzootic abortion in sheep and goats is caused by the ovine subtype of *C. psittaci* (new: *Chlamydophila abortus*). The disease has a major economic impact as it represents the most important cause of loss in sheep and goats in parts of Europe, North America, and Africa ***(6)***. This serious and potentially life-threatening zoonosis also affects pregnant women after contact with lambing ewes and leads to severe febrile illness in pregnancy ***(7,8)***.

In cattle, *C. psittaci* and *C. pecorum* were found in connection with infections of the respiratory and genital tracts, enteritis, arthritis, encephalomyelitis ***(9)***, as well as endometritis and hypofertility ***(10)***.

Chlamydioses in pigs are associated with three different species, i.e., *C. trachomatis* (new: *Chlamydia suis*), *C. pecorum* and *C. psittaci* ***(11)***. A widely held view is that chlamydiae may act in concert with other agents in multifactorial infectious diseases, such as abortions in sows, polyarthritis in piglets, diarrhea in pigs, and genital disorders in boars ***(12)***.

Other relevant animal diseases include conjunctivitis in cats caused by the feline serovar of *C. psittaci* (new: *Chlamydophila felis*), respiratory disorders and abortion in horses caused by *C. pneumoniae* (new: *Chlamydophila pneumoniae*) and *C. psittaci*, respectively. *C. pneumoniae* has also been isolated from diseased koalas and frogs.

Apart from zoonotic diseases mentioned above, chlamydiae are responsible for a number of diseases in humans, e.g., trachoma, respiratory infection, sexually transmitted infection of reproductive organs (*C. trachomatis*), as well as pneumonia in adults and cardiovascular diseases (*C. pneumoniae*).

1.2. Conventional Diagnostic Methods

As chlamydiae need to pass through an intracellular stage during their life cycle, they require tissue culture techniques to be isolated and propagated ***(13)***. Indeed, recovery of the germs through culture in suitable cell lines, such as Buffalo Green Monkey (BGM), McCoy, and HeLa, or via inoculation of embryonated hens' eggs is still regarded as the standard method in chlamydial diagnosis. Naturally, culture is an indispensable prerequisite for demonstrating the viability of a field strain, as well as for its detailed characterization by molecular and biochemical methods. There is, however, no correlation between in vitro growth and pathogenicity of an isolate. While many avian and ovine strains of *C. psittaci* (new: *Chlamydophila psittaci/abortus*) or porcine isolates of *C. trachomatis* (new: *C. suis*) can be propagated in tissue culture relatively easily, others are more difficult to grow, e.g., strains from cattle and swine belonging to *C. psittaci*. In any case, diagnosis by cell culture requires very experienced laboratory workers and standardized protocols. Results are available within 48–72 h for well-growing isolates, but may be delayed for 2–6 wk with more difficult samples.

Other methods of antigen detection include histochemical staining, e.g., Giménez stain ***(14)***, and immune fluorescence. While the methodology is relatively simple, sensitivity is lower than for culture, and as a rule, positive findings merely indicate the presence of chlamydiae in general.

Commercially available antigen enzyme linked immunosorbent assays (ELISAs) targeting chlamydial LPS only allow the detection of the genus *Chlamydia* (new: *Chlamydia* and *Chlamydophila*) without the possibility of species identification ***(15)***. Likewise, tests based on MOMP as target antigen are limited in their specificity. The attainable detection limit of 10^2–10^5 inclusion-forming units (ifu) can be insufficient for certain categories of field samples.

Table 2
Primers for Detection of Chlamydiae

Denomination	Sequence[a] (5'-3')
191CHOMP	GCI YTI TGG GAR TGY GGI TGY GCI AC
CHOMP371	TTA GAA ICK GAA TTG IGC RTT IAY GTG IGC IGC
201CHOMP	GGI GCW GMI TTC CAA TAY GCI CAR TC
CHOMP336s[b]	CCR CAA GMT TTT CTR GAY TTC AWY TTG TTR AT
218PSITT	GTA ATT TCI AGC CCA GCA CAA TTY GTG
TRACH269	ACC ATT TAA CTC CAA TGT ARG GAG TG
PNEUM268	GTA CTC CAA TGT ATG GCA CTA AAG A
204PECOR	CCA ATA YGC ACA ATC KAA ACC TCG C

[a] Degenerate nucleotides: K = G, T; M = A, C; R =A, G; W = A, T; Y = C , T; I = inosine.
[b] Modified from **ref.** ***(21)***.

Among serodiagnostic tests, the antibody ELISA is easy to handle, suitable for high sample throughput and more sensitive and faster than the complement fixation test ***(16)***. The latter is still widely used despite being rather laborious, poorly reproducible between different laboratories, and having low specificity. Generally, serological tests are based on the two main cross-reactive antigens present in all chlamydial species, LPS and MOMP, and so are not species-specific. New tests using highly specific capture antigens have been developed ***(17)***, but still need to be validated.

1.3. PCR-Based Detection and Differentiation

The possibilities of diagnostic detection of chlamydiae have considerably improved with the introduction of molecular methods, particularly the polymerase chain reaction (PCR), which permits direct identification from clinical specimens and genetically based differentiation of species.

Among the large number of tests published in the literature, only a few were designed to cover all chlamydial species. They utilize two different genomic target regions for amplification, i.e., the ribosomal RNA gene region ***(18–20)*** and the gene encoding the MOMP antigen designated *omp*1 or *omp*A ***(21,22)***. The latter harbors four variable domains known as VD I–IV and five conserved regions. Genus- and species-specific antigenic determinants are encoded by the conserved regions, and serovar-specific segments are located in VD I and VD II. This heterogeneous primary structure makes the *omp1* gene an ideal target for diagnostic PCR.

In the present chapter, a nested-PCR assay targeting the *omp*1 gene is described. The methodology is based on the paper of Kaltenböck et al. ***(21)***, only one primer was modified (*see* **Table 2**), but amplification profiles and

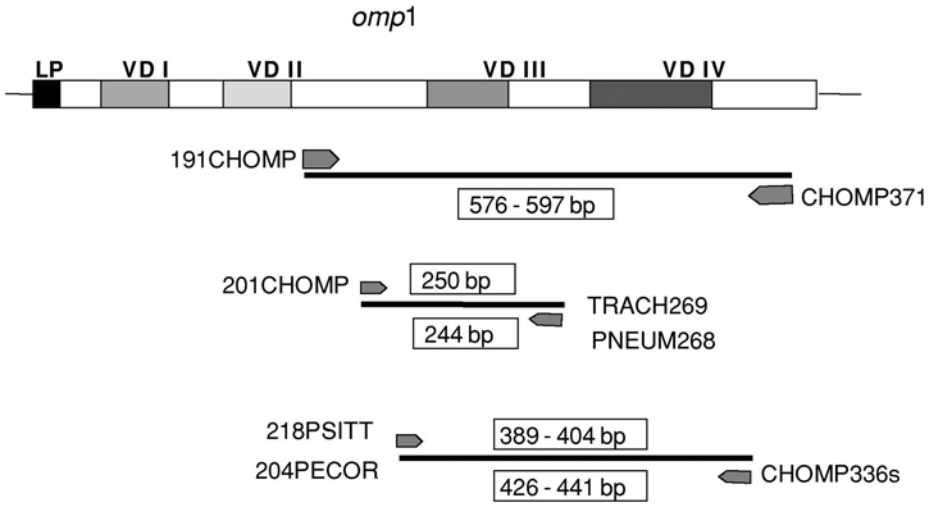

Fig. 1. Principle of detection and differentiation of chlamydial species using a nested PCR system targeting the *omp1* gene. The upper bar represents the *omp1* gene with the leader peptide (LP) and VD I–IV. In the first amplification (outer primer pair 191CHOMP/CHOMP371), a genus-specific product is generated. The second round of PCR involves one genus-specific inner primer (201CHOMP or CHOMP336s) and one species-specific primer (TRACH269/PNEUM268 or 218PSITT/204PECOR, respectively). Boxed numbers denote amplicon sizes.

pre-PCR processing of samples were optimized in our laboratory. In the first amplification, primer binding sites are located in conserved regions between VD II/VD III and downstream VD IV, whereas in the second round, primers flanking VD III (*C. trachomatis*, *C. pneumoniae*) and VD III or VD IV (*C. psittaci*, *C. pecorum*), respectively, are used. The principle of the nested amplifications and species differentiation is depicted in **Fig. 1**. Although the procedure identifies chlamydial species according to the traditional classification, we found it very robust for routine use and the most sensitive among several protocols (*see* **Note 1**).

2. Materials

2.1. DNA Extraction

1. Water. Deionized water must be used for all buffers and dilutions.
2. Lysis buffer: 100 m*M* Tris-HCl, pH 8.5, 0.05% (v/v) Tween® 20.
3. Proteinase K: 10 mg/mL in water
4. Phosphate-buffered saline (PBS): 10 m*M* Na_2HPO_4, 10 m*M* NaH_2PO_4, 145 m*M* NaCl, pH 7.0. Adjust pH by adding NaH_2PO_4.
5. Tris-EDTA (TE) buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA (ethylene diamine tetraacetic acid).
6. Sodium dodecyl sulfate (SDS) solution: 10 mg/mL in TE.

7. Phenol: saturated solution in TE buffer. If two separate phases are visible, use the lower phase only.
8. Chloroform-isoamyl alcohol: 24:1 (v/v).
9. Isopropanol, analytical, or molecular biology grade.
10. Commercially available DNA extraction kit for PCR template preparation (*see* **Note 2**). In our hands, the following products worked well: High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany), QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), E.Z.N.A. Tissue DNA Kit II (Peqlab, Erlangen, Germany).

2.2. PCR

1. *Taq* DNA polymerase. We use MasterTaq (5 U/μL) from Eppendorf (Hamburg, Germany).
2. 10X Reaction buffer for *Taq* DNA polymerase: provided by the manufacturer of the enzyme.
3. dNTP mixture: dATP + dGTP + dCTP + dTTP, 2 m*M* each. Store in aliquots at –20°C.
4. Primer oligonucleotides according to **Table 2**.

2.3. Electrophoresis and Visualization

1. Agarose, molecular biology-grade: 1% gels for PCR products of 300–1000 bp, 2% gels for products below 300 bp.
2. Tris-borate EDTA electrophoresis buffer (TBE): 0.09 *M* Tris-borate, 0.002 *M* EDTA, pH 8.0. For 1 L of 10X TBE, mix 108 g Tris-base, 55 g boric acid, and 80 mL of 0.25 *M* EDTA, make up with water. Dilute 1:10 before use.
3. Gel loading buffer (GLB): 20% (v/v) glycerol, 0.2 *M* EDTA, 0.01% (w/v) bromophenol blue, 0.2% (w/v) Ficoll® 400.
4. Ethidium bromide stock solution: 1% (10 mg/mL) solution in water.
   CAUTION: The substance is presumed to be mutagenic. Avoid direct contact with skin. Wear gloves when preparing solutions and handling gels.
5. DNA size marker. We mostly use the 100-bp DNA ladder (Gibco/Life Technologies, Eggenstein, Germany). For large fragments, *Hin*dIII-digested λ DNA (Roche Diagnostics) may be used.

2.4. General Equipment and Consumables

1. Thermal cycler. We use the T3 Thermal cycler (Biometra, Goettingen, Germany) and the Mastercycler (Eppendorf).
2. Vortex shaker, e.g., MS1 Minishaker (IKA Works, Wilmington, DE, USA).
3. Benchtop centrifuge with Eppendorf rotor, e.g., Model 5402 (Eppendorf) and/or a mini centrifuge, e.g., Capsule HF-120 (Tomy Seiko, Tokyo, Japan).
4. Heating block, for incubation of Eppendorf tubes, adjustable temperature range 30–100°C.
5. Apparatus for horizontal gel electrophoresis.
6. UV transilluminator, 254 nm and/or 312 nm.

7. Video documentation or photographic equipment.
8. Set of pipets covering the whole vol range from 0.1–1000 µL. We use the Eppendorf Research series (Eppendorf).
9. Aerosol-resistant pipet tips (filter tips),
10. Plastic tubes 0.2 or 0.5 mL, sterile, thin-walled, DNase-, and RNase-free (Eppendorf) for PCR.
11. Plastic tubes 1.5 and 2.0 mL for pre-PCR operations.

3. Methods

3.1. DNA Extraction from Different Sample Matrixes

3.1.1. Broth Culture

The simplest method to release DNA suitable for PCR from chlamydial cell cultures is 5-min boiling. After removal of cellular debris by centrifugation at 12,000*g* for 1 min, the supernatant can be used as template. Failure to amplify a specific target could be due to the presence of PCR inhibitors. In these instances, a commercial DNA extraction kit should be tried (*see* **Note 2**).

3.1.2. Swabs (e.g., Nasal, Vaginal, Conjunctival) and Mucus, Bronchoalveolar Lavage or Sputum

1. Pipet 500 µL of lysis buffer into a 2-mL Safe-Lock tube containing the cotton swab.
2. Vortex mix thoroughly for 1 min
3. Centrifuge at 12,000*g* for 30 s.
4. Put the swab into a 1-mL pipet tip whose lower half was cut off and place it all into a fresh tube.
5. Centrifuge at 12,000*g* for 1 min to press the remaining liquid out of the cotton.
6. Add the liquid to that in the first tube from **step 3**. If you have samples of mucus, bronchoalveolar lavage or sputum, start with **step 7**.
7. Centrifuge the liquid or mucus at 12,000*g* for 15 min.
8. Discard the supernatant and resuspend the pellet in 50 µL of lysis buffer.
9. Add 20 µL of proteinase K and incubate at 60°C for 2 h.
10. Inactivate the proteinase K by heating at 97°C for 15 min.
11. Centrifuge at 12,000*g* for 5 min to remove debris.
12. Use 5 µL of the supernatant for PCR.

3.1.3. Tissue from Lung, Tonsils, Lymphnodes, Spleen, Liver, and Other Organs

1. Boil 100 mg of homogenized tissue in 200 µL of water in a plastic tube for 10 min. Subsequently, allow the tube to cool to room temperature.
2. Optionally, proteinase digestion can be carried out to increase the final yield of DNA: add 200 µL SDS solution and 20 µL of proteinase K to the tube and incubate at 55°C for 1 h.
3. Add 200 µL of phenol.

4. Vortex mix vigorously for 1 min.
5. Centrifuge at 12,000*g* for 5 min.
6. Transfer the (upper) aqueous phase into a fresh tube.
7. Add 200 µL of chloroform-isoamyl alcohol.
8. Vortex mix at highest intensity for 1 min.
9. Pipet the (upper) aqueous phase into a fresh tube.
10. Precipitate DNA by adding 120 µL of isopropanol. Thoroughly mix the reagents and incubate at room temperature for 10 min.
11. Collect DNA by centrifugation at 12,000*g* for 10 min. Discard supernatant.
12. Allow DNA pellet to air-dry for 30 min.
13. Redissolve pellet in 20 µL of water. Use 1 µL for an amplification reaction.

The described procedure is the simplest method for DNA extraction from tissue specimens. Alternatively, commercial DNA extraction kits can be used (*see* **Note 2**).

3.1.4. Feces (see ***Notes 2*** *and* ***3)***

1. Add 200 µL of water to 100 mg of feces and vortex mix vigorously for 1 min.
2. Boil the suspension for 10 min.
3. Add 300 µL of phenol to each tube for DNA extraction and vigorously vortex mix the mixture for 1 min.
4. Centrifuge at 14,000*g* for 5 min.
5. Transfer the (upper) aqueous phase into fresh tubes.
6. Add 300 µL of chloroform-isoamyl alcohol to each tube.
7. Vortex mix at highest intensity for 1 min.
8. Centrifuge at 14,000*g* for 5 min.
9. Transfer the (upper) aqueous phase into a fresh tubes.
10. Add 200 µL of isopropanol for DNA precipitation.
11. Mix reagents and incubate at room temperature for 10 min.
12. Centrifuge at 14,000*g* for 10 min. Discard supernatant.
13. Allow pellet (with DNA) to air-dry for 30 min.
14. Dissolve pellet in 20 µL water. Use 1 µL in an amplification reaction.

3.1.5. Semen (see ***Note 2***)

1. Dilute 50 µL of semen in a plastic tube with 150 µL of SDS and homogenize by intensive vortex mixing.
2. Digest proteins by adding 20 µL of proteinase K solution and vortex mix for 1 min.
3. Incubate at 55°C for 1 h.
4. Continue extraction procedure as described for tissue (*see* **Subheading 3.1.3.**) beginning with **step 3**.

3.1.6. Milk Samples

We use the QIAamp DNA Stool Kit (Qiagen) according to the instructions of the manufacturer.

3.2. DNA Amplification

3.2.1. Genus-Specific Detection of Chlamydiae

1. Prepare a master mixture of reagents for all amplification reactions of the series. It should contain the following ingredients per 50-µL reaction: 1µL dNTP mixture (2 m*M* each), 1 µL primer 191CHOMP (20 pmol/µL), 1 µL primer CHOMP371 (20 pmol/µL), 5 µL reaction buffer (10X), 0.2 µL *Taq* DNA polymerase (5 U/µL), and 40.8 µL H_2O (36.8 µL in case of swab specimens).
2. Add template to each reaction vessel: 1 µL of DNA extract from infected tissue or 5 µL of extract from swab samples.
3. Include amplification controls: DNA of a chlamydial reference strain (positive control) and water (negative control 1) instead of sample extract.
4. Run PCR according to the following temperature–time profile: Initial denaturation at 95°C for 30 s, 35 cycles of denaturation (95°C for 30 s), primer annealing (50°C for 30 s), and primer extension (72°C for 30 s).
5. Correct amplification leads to the formation of a 576–597-bp product specific for the genus *Chlamydia* (according to the new taxonomy: *Chlamydia* and *Chlamydophila*).

3.2.2. Species-Specific Detection of Chlamydiae

1. Choose primer pairs according to the scheme in **Fig. 1** and **Table 2**. Prepare a master mixture of reagents for all amplification reactions of the series. It should contain the following ingredients per 50-µL reaction: 1 µL dNTP mixture (2 m*M* each), [1 µL forward primer 201CHOMP (20 pmol/µL) plus 1 µL reverse primer TRACH269 or PNEUM268 (20 pmol/µL)] or [1 µL forward primer 204PECOR or 218PSITT (20 pmol/µL) plus 1 µL reverse primer CHOMP336s (20 pmol/µL)], 5 µL reaction buffer (10X), 0.2 µL *Taq* DNA polymerase (5U/µL), and 40.8 µL H_2O.
2. Add 1 µL of the product from genus-specific PCR (*see* **Subheading 3.2.1.**) as template to each reaction vessel.
3. Subject the products of positive control and negative control 1 (1 µL of each) from the previous amplification to the second round of nested PCR. Additionally include a fresh reagent control (negative control 2).
4. Run PCR according to the following temperature–time profile: initial denaturation at 95°C for 30 s, 20 cycles of denaturation (95°C for 30 s), primer annealing (60°C for 30 s) and primer extension (72°C for 30 s), (*see* **Note 4**).
5. The correct sizes of species-specific amplicons are 250 bp for *C. trachomatis*, 244 bp for *C. pneumoniae*, 389–404 bp for *C. psittaci*, and 426–441 bp for *C. pecorum* (*see* **Fig. 1**).

The specificity of this procedure is illustrated in **Fig. 2** and discussed (*see* **Note 5**). The detection limit of the first-round PCR is in the order of 10^2 ifu, and nested amplification allowed the detection of 10^{-1} ifu (*see* **Fig. 3** and **Note 6**).

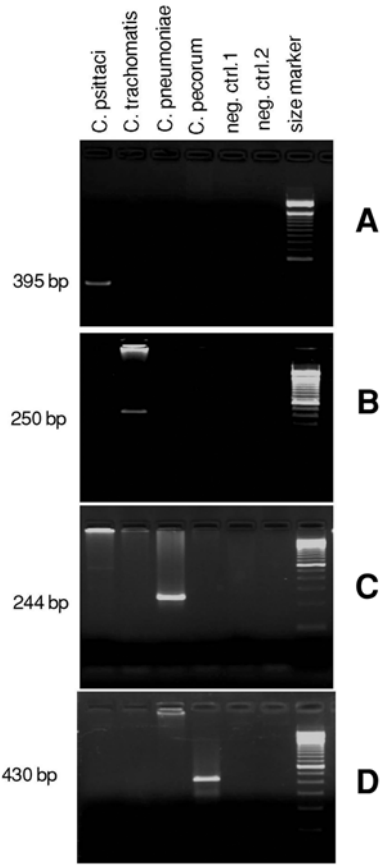

Fig. 2. Specificity of the nested amplification assay. Chromosomal DNA of field isolates of *C. psittaci*, *C. trachomatis*, *C. pneumoniae*, and *C. pecorum* was amplified according to the protocols given in **Subheadings 3.2.1.** and **3.2.2.** In the second round, the following primer pairs were used: 218PSITT/CHOMP336s **(A)**, 201CHOMP/TRACH269 **(B)**, 201CHOMP/PNEUM268 **(C)**, and 204PECOR/CHOMP336s **(D)**. Amplicon sizes are given at the left-hand margin.

3.3. Electrophoresis and Visualization

1. Prepare 1 or 2% (w/v) solution of agarose in TBE. Store gel in Erlenmeyer flasks at room temperature.
2. Liquefy gel by microwave heating (approx 30 s at 600 W) prior to use.
3. Pour gel on a horizontal surface using an appropriately sized frame.
4. Fill electrophoresis tank with TBE buffer.
5. Run the gel at a voltage corresponding to 5 V/cm of electrode distance for approx 30 min.

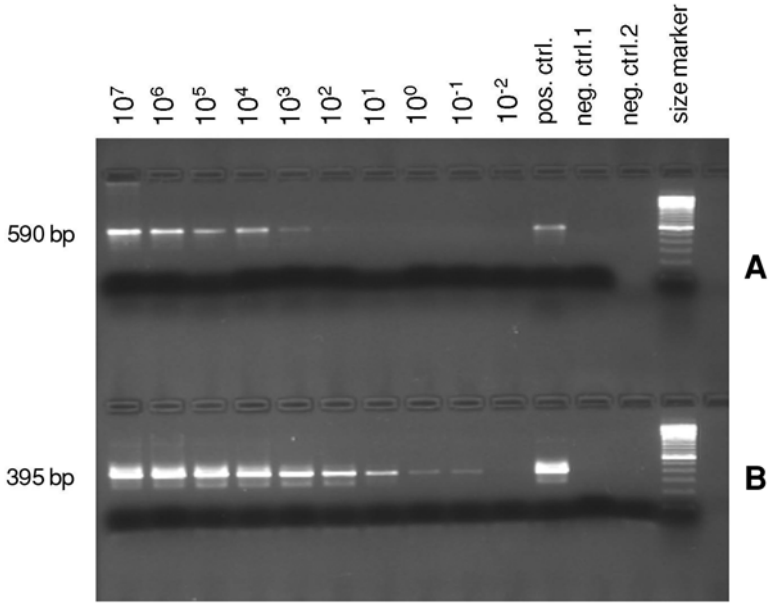

Fig. 3. Sensitivity of detection of *C. psittaci*. Chromosomal DNA was prepared from a culture of strain C5/98 of *C. psittaci* (new classification: *Chlamydophila psittaci*) in BGM cells containing 10^8 ifu/mL. Aliquots from a dilution series were amplified according to the protocols given in **Subheadings 3.2.1.** and **3.2.2.** **(A)** Genus-specific amplification using primer pair 191CHOMP/CHOMP371. **(B)** Nested PCR using primers 218PSITT/CHOMP336s in the second round.

6. Load each well with 10 µL of PCR product mixed with 5 µL GLB.
7. Stain DNA bands by immersing the gel in ethidium bromide solution containing 5 µL of stock solution in 200 mL of water. (Alternatively, ethidium bromide-containing agarose gels can be used. Add 5 µL of ethidium bromide solution to 100 mL of melted agarose in TBE buffer.)
8. Visualize bands under UV light using a transilluminator.

4. Notes

1. For differentiation of species according to the new taxonomic classification, the method of Everett and Andersen ***(19)*** is recommended, which involves amplification of a approx 600-bp fragment of the 16–23S intergenic spacer region of the rRNA operon with subsequent restriction enzyme analysis. However, the method is less sensitive than the present *omp*1-PCR by two orders of magnitude ***(23)***, which makes it unsuitable for most field samples. In our hands, its main area of application is the identification and differentiation of cultured strains.
2. The use of commercial DNA preparation kits can be recommended for samples of organ tissue, broth culture, and, with some qualification, also for semen and feces.

In the latter instances, the kit should be tested with a series of spiked samples containing defined numbers of chlamydia cells in order to examine its suitability.

Most commercial kits are easy to work with. They contain a special buffer reagent for lysis of the bacterial and tissue cells, the effectiveness of which is decisive for the kit's performance. An optional RNase digestion is intended to remove cellular RNA. The lysate is then centrifuged through a mini-column, where the released DNA is selectively bound to a solid phase (modified silica, hydroxyl apatite, or special filter membrane). After washing, the DNA can be eluted with an elution buffer or water. DNA prepared in this manner is usually of high purity and free of PCR inhibitors.

It should be noted, however, that the yield of extracted DNA is limited by the binding capacity of the mini-column. If maximum recovery of chlamydial DNA from high-titer samples is important, e.g., in quantitative assays or for preparation of reference DNA, one of the multi-step extraction protocols should be followed.

3. Commercial DNA extraction kits for human stool specimens can be used in principle. In our hands, the QIAamp DNA Stool Kit and the Invitek Spin Stool DNA Kit (InVitek, Berlin, Germany) worked reasonably well. However, chlamydial DNA recovery from specimens containing less than 10^3 ifu proved difficult. For these cases, we recommend phenol-chloroform extraction as described in **Subheading 3.1.4.**
4. Practical experience from routine use of the present methodology led us to set the number of cycles to 20 in the second round of amplification. Running 30 cycles or more would yield an increase in sensitivity, but the nested assay would be more vulnerable to carryover contamination.
5. To assess the performance of the present PCR assay, a validation study was conducted in our laboratory ***(23)***. In a series of 99 samples, PCR proved more sensitive than cell culture, whereas both methods were nearly equivalent in terms of specificity. The figures for cell culture were: sensitivity 74%, specificity 96%.
6. For correct interpretation of the sensitivity figures, it has to be noted that the count of ifu is representative of the number of elementary bodies, but does not comprise all chlamydial cells present in the culture or sample. Reticular bodies residing in cellular inclusions also provide target DNA. Consequently, a content of 10^{-1} ifu in infected cell culture or tissue does not necessarily mean that there is less than one chlamydial cell.

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