Polymerase Chain Reaction for Detection of *Chlamydia pneumoniae* in Gargled-Water Specimens of Children

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The objective of the present study was to establish the occurrence of *Chlamydia pneumoniae* by direct detection in gargled-water specimens obtained from 193 children suffering from acute or chronic respiratory infections. Specimens were analyzed by an indirect immunofluorescence test (IIF), a genus-specific antigen enzyme immunosorbent assay (EIA) and the polymerase chain reaction (PCR). The pathogen was detected in three children by PCR only. As underlying disease, chronic obstructive bronchitis resistant to therapy was reported. In two of the children, the presence of pneumonia could be verified by X-ray. With a detection threshold of target DNA obtained from two inclusion forming units (IFU), the PCR proved clearly more sensitive than EIA becoming positive at levels of 100 IFU and above. No interpretable results could be obtained for the IIF.

Chlamydia pneumoniae is the third species of the genus *Chlamydia*. It has recently been described and is thought to cause various infections of the upper and lower respiratory tract (1–8). Several well-documented studies have reported a high prevalence of this pathogen in the adult population (2, 4). In contrast, only few data from primarily serological investigations are available for assessing its importance during childhood (9–13). The aim of this study was to establish the occurrence of *Chlamydia pneumoniae* in children with respiratory infections by means of direct detection methods.

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Patients and Methods. A total of 193 children aged five to 16 years (mean age: 10 years; 113 males, 80 females) with acute or chronic respiratory diseases were enrolled in the study. Twenty-five of the children (17 aged 6–10 years, 8 aged 11–16 years) were known to be asthmatics; ten children showed a manifest pneumonia (7 aged 6–10 years, 3 aged 11–16 years). The remaining 158 children (25 aged 0–5 years, 102 aged 6–10 years, 31 aged 11–16 years) suffered from chronic obstructive bronchitis. The actual symptoms were cough, sore throat with reddened pharynx and pyrexia of up to 39°C.

There were only children enrolled in the study whose parents gave their explicit consent. For the detection of *Chlamydia pneumoniae* gargled water was taken as specimen. After quickly rinsing their mouths with tap water, the children were told to gargle with approximately 30 ml of collection liquid for 30 seconds with the head reclined. As 13 of the children were too young for gargling, three pharyngeal swabs were taken. Until transfer to the laboratory, the samples were stored at 4°C. At the laboratory, the specimens were centrifuged (3000 g, 15 min), washed, resuspended in 400 µl phosphate buffered saline (PBS, pH 7.4) and stored at -20°C.

For IIF, 20 µl of a resuspended gargled-water pellet was applied to a slide (Syva-Mikrotrak, Merck, Germany), or the pharyngeal swab was rolled back and forth on the slide. Then both types of specimens were air dried, acetone fixed (5 min) and immediately analysed using a species-specific, monoclonal fluorescent antibody test (Cellabs Diagnostic, Australia). Serial dilutions from a control strain of *Chlamydia pneumoniae* (AR 388, Washington Research Foundation, USA) were prepared in PBS (pH 7.4) containing 10^1 to 10^5 inclusion forming units (IFU)/µl and served as positive controls.

Fifty µl of the resuspended pellet or a throat swab was placed into CELISA transport medium and tested with a genus-specific antigen EIA (Cellabs Diagnostic). The sensitivity of the EIA was tested using dilutions of the control strain AR 388.

For PCR, a resuspended pellet of gargled water (approximately 300 µl) was mixed with 200 µl PBS (pH 7.4) or a throat swab and eluted in 0.5 ml PBS (pH 7.4). These samples were boiled for 10 min at 100°C, frozen at -20°C, thawed and refrozen. After a further thawing cycle at 100°C, the specimen was centrifuged (3000 g; 10 min) and DNA prepared according to the method described by Sambrook et al. (14). Controls for the

detection of PCR inhibitors in clinical specimens were prepared as follows. For each of ten clinical specimens, 10^2 IFU of the control strain AR 388 was placed into 0.5 ml PBS (pH 7.4) and processed as described above. The obtained extract was mixed with the extracts of ten specimens (5 µl each) so that the final PCR volume contained DNA extracted from 2 IFU of *Chlamydia pneumoniae*. Adequate positive and negative controls were run in each assay.

Using the primer-pair CpnA/CpnB, PCR was done as previously described by Gaydos et al. (15). The final mixture contained 0.5 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate, 1 x PCR buffer (10 mM Tris, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 0.01 % gelatin), and 2.5 U Taq-polymerase (all from Perkin-Elmer Cetus, USA).

Amplification was performed in a thermal cycler (Biometra, Germany) with 50 repetitive cycles. Denaturation of DNA was done at 94°C for 5 min for cycle 1 and 1 min for cycles 2 to 50. Primer annealing occurred at 55°C for 1 min and primer extension at 72°C for 1 min. An additional primer extension at 72°C for 5 min followed the last cycle. In contrast to Gaydos et al. (15), another 2.5 U of Ampli-Taq DNA-polymerase were added to the reaction mixtures after cycle 30. Positive PCR results were confirmed by using two further primer pairs, HL1/HR1 and HM1/HR1 (16). Amplification products were separated by agarose gel electrophoresis using TRIS-borate-EDTA buffer (pH 8.3). Nucleic acids were then visualized by staining with ethidium bromide. The PCR was regarded as positive for each of the primer pairs used if the product had the expected molecular mass.

Results and Discussion. The present study was intended to assess the percentage of children actually suffering from respiratory infections due to *Chlamydia pneumoniae*. For this purpose, direct methods for the detection of the causative organism are required. To our knowledge, the rather costly chlamydial cell culture and the immunofluorescence test, the objectivity of which is controversial, have been the only methods tried in children to date (8,17,20).

While in most investigations throat swabs were used, we decided to use gargled-water specimens because they were shown to have a higher cell content. A comparison of the epithelial cell count in gargled-water solutions and pharyngeal swabs from 20 healthy volunteers showed a significantly higher cell count in gargled water (average count of 4×10^5 and 7×10^4 epithelial cells, respectively; Wilcoxon test, 2 $p \leq 0.001$). In addition, it would yield investigation material of sufficient quality and quantity for three tests. Collection of gargled-water specimens was associated with a higher patient acceptance.

With PCR, *Chlamydia pneumoniae* could be detected in three of the 193 (1.5 %) children. With the primers CpnA/CpnB, the DNA extracts prepared from specimens of these children yielded products of the expected molecular mass (463 bp) after amplification. With the primers HL1/HR1, products of 437 bp were seen and another primer pair, HM1/HR1, yielded products of 229 bp (Figure 1).

All PCR-positive children were male. They were six and a half, seven and eight years old and reportedly suffered from chronic obstructive bronchitis which had proved resistant to antimicrobial chemotherapy. The actual signs and symptoms causing the parents to consult the outpatient department of a children's hospital were cough, sore throat with reddened pharynx and pyrexia of up to 39°C. In two of the children, pneumonia was verified by X-ray. Thus *Chlamydia pneumoniae* was found in two out of ten children with pneumonia.

Additionally, we used a new IIF which became available recently, as well as a genus-specific antigen-EIA. EIA was used because a genus-specific test could at least be useful as a screening test.

Due to numerous unspecific reactions, we were not able to interpret the results of the IIF. This is consistent with the findings of other authors, who either found a surprisingly high proportion of positives (7) or reported culture-positive speci-

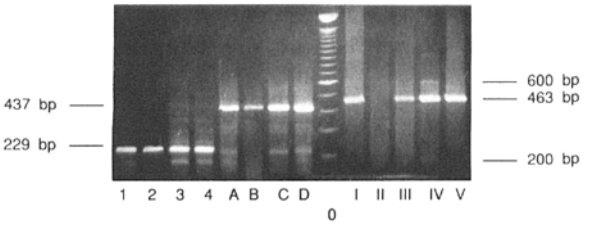

Figure 1: Ethidium bromide-stained agarose gel showing PCR-products of three *Chlamydia pneumoniae* positive patients (Lane 2, B, III: patient C.C.; Lane 3, C, IV: patient J.J.; Lane 4, D, V: patient B.M.) with primer pair HM1/HR1 (2–4), HL1/HR1 (B–D) and CpnA/CpnB (III–V). Lane 1, A, I: *Chlamydia pneumoniae*-DNA (~ 20 IFU) amplified as positive control. Lane II: negative control. Lane 0: 100 bp molecular weight marker.

mens being negative by the IIF (19). The Cellabs IFA has not been systematically evaluated for this use, and for this reason the lack of sensitivity and specificity is not surprising. Its use should be limited to culture confirmation.

EIA was negative in all clinical specimens. This may be due to the higher detection threshold (100 IFU/100 µl) as compared to that for the PCR (target DNA of 2 IFU). These results are not surprising; when Chirgwin et al. (8) did NP Chlamydiazyme, the EIA detected only two of 15 culture-positive patients. A possible explanation could be that mucus, food remnants or organisms of the oral and pharyngeal flora unspecifically bind to the "catcher"-antibodies coating the bottom of the wells in the microtiter plate. In addition, the specimen volume tested in the EIA is smaller than that processed for the PCR. Consequently, chlamydial elements are more likely to escape detection.

A possible disadvantage of this study is that cultures were not performed. A golden standard for the diagnosis of acute *Chlamydia pneumoniae* infections could not yet be recommended. Some of the data available (3, 21) indicate that diagnosis of acute infections with *Chlamydia pneumoniae* is more reliable and expedient by means of PCR. Campbell et al. (16) examined throat swabs of 36 persons, eight of whom were positive by both culture and PCR. In addition PCR was positive in four culture-negative patients. This finding is consistent with the results of Gaydos et al. (15) who could demonstrate the presence of the bacterium by PCR in five of 98 culture-negative throat swabs. In another study, Gaydos et al. (22) found culture more sensitive than PCR.

Our results indicate that *Chlamydia pneumoniae* must be regarded as a potential although not a common cause of respiratory infection in children.

A test such as PCR which allows for the rapid detection of the pathogen may be helpful in determining the correct diagnosis initially and thus enable selection of an adequate chemotherapy. As a consequence, the eradication of *Chlamydia pneumoniae* may prevent the development of a chronic chlamydial infection in childhood.

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